The Struggle for Iron: How it Affects Cooperation, Competition and Biodiversity in Bacterial Communities

Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich

von

Anne Leinweber

aus
Deutschland

Promotionskommission

Prof. Dr.Rolf Kümmerli (Vorsitz)

Prof. Dr. Leo Eberl

Prof. Dr. Martin Ackermann

Zürich, 2018

Table of Contents

Abbreviations

AHL	Acyl-homoserine lactone (QS signals)
BC	*Burkholderia cenocepacia*
BDSF	*Burkholderia* DSF (QS signal)
cDNA	Copy-DNA
CF	Cystic fibrosis
DSF	Diffusible signal factor
FDR	False discovery rate after Benjamini and Hochberg 1995
FU	Fluorescence units
Fur	Ferric uptake regulator
GSEA	Gene set enrichment analysis
HCN	Hydrogen cyanide
NES	Normalized enrichment score (test statistic of GSEA)
OD600	Optical density at a wavelength of 600 nm
orb	ornibactin
PA	*Pseudomonas aeruginosa*
PCA	Principal component analysis
pch	pyochelin
PQS	*Pseudomonas* quinolone signal (QS signal)
pvd	Pyoverdine
QS	Quorum sensing
rlog	Regularized logarithm
rRNA	Ribosomal RNA
SN	Supernatant
T6SS (T1SS, T2SS, T3SS, T5SS)	Type-6-secretion system
TPS system	Two-partner-secretion system

Summary

Bacteria are ubiquitous organisms that usually live in multispecies communities, where they interact with other microbes and organisms of other kingdoms. In these communities, microbes share common resources and space, which leads to high competition, but also to cooperation, within and between species. One of the best studied examples of cooperative behavior is the communal iron uptake. Bacteria secrete small peptides, so-called siderophores, to scavenge iron from the environment. These siderophores function as public goods that can be taken up by all members of the population that carry the respective receptor. However, this metabolically costly cooperation can be exploited. Siderophore non-producers can evolve that do not contribute to the production of public goods anymore, but still take up siderophores produced by others. Hence, these non-producers have a fitness advantage over the producers and can spread in the population. Therefore, competition in microbial communities can happen between and within species. Competition can manifest in two types: direct interference and indirect resource competition, where bacteria either directly harm their opponents (e.g. through toxins), or increase their resource consumption without harming competitors, respectively. These types of competition are not mutually exclusive but can be employed simultaneously. In order to express a competitive phenotype, bacteria need to be able to sense the presence of competitors. One possibility is that bacteria directly respond to cell-cell contact or to compounds that are derived from competitors (e.g. toxins, secreted enzymes). Alternatively, bacteria can indirectly respond to ecological stress imposed by competitors, like resource limitation or oxidative stress.

In my thesis, I address the question how within and between species competition influence the individual bacteria and the multispecies community as a whole. For that purpose, I used a model community consisting of *Pseudomonas aeruginosa* (PA) and *Burkholderia cenocepacia* (BC). Both species occupy the same habitats ranging from soil over aquatic to marine habitats and infect immunocompromised patients (e.g. lungs of cystic fibrosis patients). In these habitats, iron availability is usually limited, which is why bacteria need to actively take it up from the environment. One way to scavenge iron is the secretion of highly iron affine siderophores. PA and BC secrete unique primary siderophores, pyoverdine and ornibactin, respectively, which function as public goods within their own populations and cannot be exchanged between the two species (Meyer et al. 1989). In my thesis, I focused on the competition for iron between PA and BC that is mediated by these siderophores.

In my first project, I investigated the effect of spatial structure, iron availability and within species competition on the coexistence of PA and BC. PA and BC are regularly found in the same natural habitats. However, in the laboratory it is challenging to establish stable cocultures, since PA regularly outcompetes BC in planktonic cocultures. I found that the presence of a PA siderophore non-producer

can facilitate coexistence of PA and BC in planktonic cocultures by increasing the within species competition, thereby lowering the competition between PA and BC. This “cheating effect” could explain the high biodiversity that is frequently found in habitats with low spatial structure, like marine environments. Furthermore, my first project showed that BC was outcompeted by the PA siderophore producer as well as the PA siderophore non-producer in two-way competitions under high iron availability, indicating that the competition between PA and BC is not only mediated by siderophores.

Therefore, in my second project, I investigated the competition between PA and BC further. I wanted to know, if PA changes the expression of pyoverdine when it is competing with BC for iron and if PA can adjust this phenotype gradually to match different levels of inter-species competition. I also investigated the fitness consequences of phenotypic plasticity in PA. Fine-tuned phenotypic adjustments in response to different levels of competitions are well known in higher organisms that show competitive behavior only when it is necessary and that is further adjusted to match the prevailing level of competition. I found similar levels of fine-tuned phenotypic responses in the bacterial model community of PA and BC. Here, PA increases its early pyoverdine synthesis due to competition with BC. This generates a fitness advantage to PA in the form of an earlier initiation of growth relative to PA monocultures. I observed that these phenotypic changes are gradually adjusted to match the prevailing level of competition by BC. However, I found evidence that the early fitness advantage due to an increased pyoverdine synthesis contributed to trade-offs that lead to fitness disadvantages during later competition stages, in the form of reduced growth rate and yield. While increased pyoverdine synthesis and changed early growth phenotypes only occurred under iron restriction, when pyoverdine is essential for iron uptake, I found that PA’s growth rate and yield were also reduced in the mixed cultures with BC under iron repletion. This indicates that traits other than pyoverdine influence the competition between PA and BC, as I have also seen in project 1.

In my third project, I wanted to investigate, which traits are involved in the competition of PA against BC, to understand PA’s dominance in the mixed community (project 1) and to find out, which traits could contribute to fitness trade-offs in PA (project 2). Therefore, I investigated PA’s global response to its competitor BC. In particular, I was interested in whether PA can sense diffusible signals of BC and mount defensive responses towards or if it prepares to directly attack its competitor. I therefore grew PA in a mixture of fresh media and spent BC culture supernatant containing diffusible cues of BC. I used PA grown in its own supernatant and in unconditioned medium as reference treatments. By using RNA sequencing, I measured the expression levels of genes encoding traits known to be involved in either defense or attack mechanisms. I therefore manually compiled 57 gene sets from the literature. These traits involve biofilm formation, motility, quorum sensing, siderophore-mediated iron uptake, secretion systems, secreted enzymes and secondary metabolites. I found that diffusible cues from BC

lead to substantial gene expression changes in PA, indicating that signals from a distant competitor, and not only direct cell-cell contact, are sufficient to express a competitive phenotype. My results show that PA initiates a complex multivariate response in response to diffusible cues from its competitor, simultaneously regulating several traits. In addition, I found that the iron availability strongly influences PA's response. Only a few traits are regulated in response to BC derived signals regardless of the iron availability. The HHQ synthesis, a precursor molecule for the PQS signal (part of a PA quorum sensing system) that also has antibacterial properties, was the only mechanism that was upregulated in low and high iron availability alike in response to BC compounds. I found that the biofilm formation, AHL based quorum sensing systems and the type-3-secretion system were downregulated or not affected in response to BC derived cues under low and high iron availability. When iron was limited, BC-derived signals lead to the activation of few but highly toxic traits, the hydrogen cyanide and phenazine synthesis. Under high iron availability, PA invested in a broader spectrum of activities. Genes encoding flagella assembly, type-6-secretion system assembly and secreted toxins, and the pyocin and pyoverdine synthesis were upregulated. The genes of hydrolyzing enzymes were regulated differently demonstrating that they are controlled by different regulatory circuits. In summary, my third project showed that under low iron availability PA activates an attacking mode against BC, whereas it initiates a broader response, including swimming motility (flagella), iron uptake (pyoverdine), toxins (pyocins) and contact dependent inhibition (T6SS), when iron is not restricted. These mechanisms could explain PA's dominance over BC in a range of iron availabilities that I have observed throughout my experiments and could contribute to fitness trade-offs.

In conclusion, the results of my projects demonstrate the large influence of the environment on the competition between two bacterial species. I varied iron availability and spatial structure, which lead to changes of the competitive phenotype and fitness of the dominant competitor PA (project 2). Further, the environment determined the competitive means that are initiated by PA in response to inter-specific competition (project 3). The level of within-species competition was also strongly influenced by environmental conditions, which can mediate biodiversity of my model community (project 1). Thus, environmental factors determine the type of social interactions within and between bacterial species and can directly influence biodiversity.

Zusammenfassung

Bakterien sind allgegenwärtig und Leben oftmals in komplexen Gemeinschaften, bestehend aus anderen Mikroben und Arten anderer Reiche, wie Pflanzen und Tiere. In diesen Gemeinschaften teilen Mikroben den gemeinsamen Lebensraum und Ressourcen, was zu hoher Konkurrenz, aber auch zu Kooperation führen kann, sowohl zwischen als auch innerhalb von Arten. Eines der am besten untersuchten Beispiele kooperativen Verhaltens ist die gemeinschaftliche Eisenaufnahme. Dafür sekretieren Bakterien Peptide, sogenannte Siderophore, um Eisen aus der Umgebung aufzunehmen. Diese Siderophore fungieren als öffentliche Güter, die von allen Mitgliedern der Population aufgenommen werden können, die den entsprechenden Rezeptor tragen. Diese metabolisch kostspielige Kooperation kann jedoch ausgenutzt werden. Nicht-Produzenten von Siderophoren können sich entwickeln, die aufhören zur Produktion der öffentlichen Güter beizutragen, aber weiterhin die Siderophore von den Produzenten aufnehmen. Dadurch erhalten diese Nicht-Produzenten einen Fitnessvorteil gegenüber den Produzenten und können sich in der Population ausbreiten. Dieses Beispiel zeigt, dass Kooperation immer auch zu Konkurrenz zwischen nahverwandten Stämmen führen kann. Konkurrenz im allgemeinen kann in zwei Kategorien aufgeteilt werden.: direkte beeinträchtigende und indirekte ausbeuterische Konkurrenz. Bei ersterer fügen Bakterien ihren Gegnern direkt Schaden zu (z. B. durch Toxine). Bei der zweiten Art erhöhen Bakterien ihren Ressourcenverbrauch, ohne ihre Gegner zu verletzen. Diese Arten von Konkurrenz schliessen sich nicht gegenseitig aus, sondern können gleichzeitig zum Zuge kommen. Um einen kompetitiven Phänotyp zu zeigen, müssen Bakterien allerdings in der Lage sein, die Anwesenheit von Konkurrenten zu bemerken. Eine Möglichkeit ist, dass sie direkt auf den Zell-Zell-Kontakt oder auf Moleküle, die von Konkurrenten stammen (z. B. Toxine, sekretierte Enzyme), reagieren. Andererseits können sie auf ökologischen Stress reagieren, der durch andere Bakterien hervorgerufen werden wird, wie Ressourcenlimitierung oder oxidativen Stress.

Die Frage ist, wie Wettkampf zwischen und innerhalb von Arten einzelne Bakterien und die Gemeinschaft als Ganzes beeinflusst. In meiner Doktorarbeit habe ich genau diese Fragen untersucht. Dazu habe ich ein Modell-System entwickelt, welches aus den Bakterien *Pseudomonas aeruginosa* (PA) und *Burkholderia cenocepacia* (BC) besteht. Beide Arten leben in den gleichen Lebensräumen, die von Boden- über aquatische bis zu marinen Habitaten reichen. Beide Arten können auch immungeschwächte Patienten infizieren (z. B. Lungen von Mukoviszidose-Patienten). In diesen Lebensräumen ist die Eisenverfügbarkeit in der Regel begrenzt, weshalb Bakterien Eisen aktiv aus der Umwelt aufnehmen müssen. Eine Möglichkeit dafür ist die Sekretion von stark eisenaffinen Siderophoren. PA und BC sekretieren spezifische primäre Siderophore, Pyoverdin und Ornibactin, die als öffentliche Güter innerhalb ihrer eigenen Populationen fungieren und nicht zwischen den beiden

Spezies ausgetauscht werden können. In meiner Doktorarbeit habe ich mich auf den Wettkampf um Eisen zwischen PA und BC konzentriert, der durch Siderophore ausgetragen wird.

In meinem ersten Projekt habe ich den Einfluss von räumlicher Struktur, Eisenverfügbarkeit und Wettkampf innerhalb einer Spezies auf die Koexistenz von PA und BC untersucht. PA und BC werden regelmässig in gleichen natürlichen Lebensräumen gefunden. Im Labor ist es jedoch schwierig, stabile Gemeinschaften zu etablieren, da PA in planktonischen Kokulturen BC in der Regel verdrängt. Ich fand heraus, dass das Vorhandensein eines PA Stammes, welcher keine Siderophore mehr produziert, die Koexistenz von PA und BC in planktonischen Kokulturen ermöglichen kann. Der Grund dafür ist, dass der Siderophore-Nichtproduzent durch Ausbeutung der Siderophore die Konkurrenz innerhalb von PA Stämmen erhöht, und dadurch die Konkurrenz zwischen PA und BC verringert. Dieser "Betrugseffekt" könnte die hohe Biodiversität erklären, die oft in Lebensräumen mit geringer räumlicher Struktur, wie marinen Gewässern, vorkommt. Darüber hinaus zeigte mein erstes Projekt, dass BC sowohl vom PA-Siderophorproduzenten als auch vom PA-Siderophor-Nichtproduzenten in Zweierwettkämpfen unter hoher Eisenverfügbarkeit unterdrückt wurde. Dies deutet darauf hin, dass die Konkurrenz zwischen PA und BC nicht nur durch Siderophore verursacht wird.

Daher habe ich in meinem zweiten Projekt den Wettkampf zwischen PA und BC weiter untersucht. Ich wollte wissen, ob PA die Expression von Pyoverdin verändert, wenn es mit BC um Eisen konkurriert und ob PA diesen Phänotyp sukzessive an verschiedene Stufen des Konkurrenzdrucks einer anderen Spezies anpassen kann. Ich habe zudem die Fitnesskonsequenzen für PA, welche durch phänotypische Plastizität entstehen, untersucht. Feinabgestimmte phänotypische Anpassungen als Reaktion auf unterschiedliche Stufen von Konkurrenzdruck sind von höheren Organismen bekannt, die nur dann kompetitives Verhalten zeigen, wenn es notwendig ist und dieses auch an das vorherrschende Intensität des Wettkampfes anpassen. Ich habe ein vergleichbares Bild in der Bakteriengemeinschaft bestehend aus PA und BC gefunden. Hier produziert PA zunächst mehr Pyoverdin als Antwort auf die Konkurrenz mit BC. Dies bringt einen Fitnessvorteil für PA, der im Vergleich zu PA-Monokulturen zu einer früheren Initiierung des Wachstums führt. Es stellte sich heraus, dass PA diese phänotypischen Veränderungen stufenweise an den vorherrschenden Konkurrenzdruck durch BC anpasst. Meine Ergebnisse deuten zudem an, dass der frühzeitige Fitnessvorteil aufgrund der erhöhten Pyoverdinsynthese zu einem evolutionären Kompromiss beitragen könnten, welcher zu Fitnessnachteilen in späteren Wettbewerbsstadien führt. Diese Fitnessnachteile manifestierten sich in einer geringeren Wachstumsrate und einem niedrigeren Wachstumsertrag. Die erhöhte Pyoverdinsynthese und damit auch der veränderte frühe Wachstumsphänotyp haben sich jedoch nur unter Eisenlimitation gezeigt, wenn Pyoverdin essentiell ist für die Eisenaufnahme. Im Gegensatz waren die Wachstumsrate und der Wachstumsertrag von PA auch unter Eisensättigung in den

gemischten Kulturen mit BC reduziert. Dies deutet darauf hin, dass ausser Pyoverdin auch andere Mechanismen die Konkurrenz zwischen PA und BC beeinflussen, wie auch schon Projekt 1 gezeigt hat.

In meinem dritten Projekt wollte ich untersuchen, welche Mechanismen den Wettkampf zwischen PA und BC beeinflussen, um die Dominanz von PA in der gemischten Kulturen besser zu verstehen (Projekt 1) und um herauszufinden, welche Merkmale zu Fitness-Kompromissen in PA beitragen könnten (Projekt 2). Daher habe ich die globale Reaktion von PA auf seinen Konkurrenten BC untersucht. Insbesondere war ich daran interessiert, ob PA Bakterien diffusionsfähige Signale von BC wahrnehmen können und daraufhin eine defensive Reaktion initiieren, oder ob sie sich darauf vorbereiten, den Konkurrenten direkt anzugreifen. Ich habe daher PA in einer Mischung aus frischem Medium und verbrauchtem BC-Kulturüberstand kultiviert, der diffusionsfähige Signale von BC enthielt. Als Referenzen dienten PA, welche entweder in ihrem eigenen Überstand oder in unbehandeltem Medium kultiviert wurden. Durch RNA-Sequenzierung habe ich die Expressionslevel von Genen gemessen, die Mechanismen kodieren, von denen bekannt ist, dass sie an Verteidigungs- oder Angriffsstrategien beteiligt sind. Ich habe dafür 57 Gensätze aus der Literatur manuell zusammengestellt. Diese Mechanismen umfassen Biofilmbildung, Motilität, Quorum Sensing, Siderophor vermittelte Eisenaufnahme, Sekretionssysteme, sekretierte Enzyme und die Produktion von sekundären Metaboliten.

Ich fand heraus, dass diffusionsfähige Signale von BC zu wesentlichen Veränderungen der Genexpression in PA führen, was darauf hindeutet, dass Signale von einem räumlich entfernten Konkurrenten und nicht nur der direkte Zell-Zell-Kontakt ausreichen, um einen kompetitiven Phänotyp zu exprimieren. Meine Ergebnisse zeigen, dass PA eine komplexe multivariate Antwort als Reaktion auf diffundierende Signale von einem Konkurrenten initiiert und so gleichzeitig die Expression mehrerer Mechanismen reguliert. Ausserdem habe ich festgestellt, dass die Eisenverfügbarkeit einen starken Einfluss auf die Antwort von PA hat. Nur wenige Mechanismen werden unabhängig von der Eisenverfügbarkeit als Reaktion auf BC-Signale reguliert. Die HHQ-Synthese, ein Vorläufer-Molekül für das PQS-Signal (Teil eines PA-Quorum-Sensing Systems), das ebenfalls antibakterielle Eigenschaften aufweist, war der einzige Mechanismus, dessen Expression in Reaktion auf BC-Verbindungen in niedriger und hoher Eisenverfügbarkeit stimuliert wurde. Die Biofilmbildung, AHL-basierte Quorum-Sensing Systeme und das Typ-3-Sekretionssystem wurden als Reaktion auf BC-Signale bei niedriger und hoher Eisenverfügbarkeit herunterreguliert oder nicht beeinflusst. Wenn Eisen begrenzt war, führten BC-Signale zur Aktivierung von wenigen, aber sehr toxischen Mechanismen: die Synthese von Cyanwasserstoff und Phenazinen. Bei hoher Eisenverfügbarkeit investierte PA in ein breiteres Spektrum von Mechanismen. Gene wurden hochreguliert, die den Zusammenbau von Flagella und des Typ-6-Sekretionssystems, die Synthese von Pyocinen und Pyoverdin, sowie sekretierte Toxine

kodieren. Die Gene von hydrolysierenden Enzymen wurden unterschiedlich reguliert, was zeigt, dass diese durch verschiedene regulatorische Schaltkreise gesteuert werden. Zusammenfassend zeigt mein drittes Projekt, dass PA bei geringer Eisenverfügbarkeit einen Angriffsmodus gegen BC aktiviert, während es eine breiter aufgestellte Reaktion einleitet, wenn die Verfügbarkeit von Eisen nicht beschränkt ist. Diese Antwort schliesst die Schwimmmotilität (Flagella), Eisenaufnahme (Pyoverdin), Toxine (Pyocine) und kontaktabhängige Inhibitionsmechanismen ein. Diese Mechanismen könnten die Dominanz von PA über BC in einer Reihe von Eisenverfügbarkeiten erklären (Projekt 1) und weiterhin zu Fitness-Kompromissen beitragen (Projekt 2), die ich während meiner Experimente beobachtet habe.

Abschliessend zeigen die Ergebnisse meiner Projekte den grossen Einfluss von Umweltfaktoren auf den Wettkampf zwischen zwei Bakterienspezies. Ich habe die Eisenverfügbarkeit und die räumliche Struktur in meinen Experimenten variiert, was zu Veränderungen des kompetitiven Phänotyps und der Fitness der dominanten Spezies PA führte (Projekt 1 und 2). Darüber hinaus bestimmte die Umwelt die kompetitiven Mechanismen, die von PA als Reaktion auf den Wettkampf zwischen Spezies initiiert wurden (Projekt 3). Die Stärke des Wettkampfes innerhalb derselben Spezies wurde ebenfalls stark von Umweltbedingungen beeinflusst, was wiederum die Diversität meines Modells einer Bakteriengesellschaft beeinflussen konnte (Projekt 1). So bestimmen Umweltfaktoren die Art der sozialen Interaktionen zwischen und innerhalb von Bakterienspezies und können somit direkt die Biodiversität beeinflussen.

1. General Introduction

1.1. Cooperation and competition in natural bacterial communities

1.1.1. Conceptual framework of social behaviors

For a long time, bacteria have been seen as solitary individuals that do not interact with one another. But this view has changed during the past years. Bacteria are indeed highly social, interacting with other bacteria and individuals from other kingdoms.

Well-known examples of social interactions in bacteria are the cell-to-cell communication by quorum sensing (QS) signals, the formation of biofilms and the cooperative secretion of iron-scavenging siderophores. Bacteria secrete specific QS signals to synchronize the gene expression on the population level. Thus, QS is a way to coordinate population behavior (Juhas et al. 2005; Girard and Bloemberg 2008; Subramoni and Sokol 2012; Schuster et al. 2013; Fazli et al. 2014; Lee and Zhang 2014; Goo et al. 2015). One QS controlled trait is the secretion of extracellular matrix molecules that form the scaffold of biofilms. Biofilms can be either free floating or attached to surfaces and are a common natural lifestyle of bacteria (Flemming et al. 2016; Nadell et al. 2016). Bacteria also collectively secrete siderophores, small peptides with a high iron affinity to scavenge this essential nutrient from the environment (Buckling et al. 2007; Harrison and Buckling 2009; Lujan et al. 2015). But bacteria also engage in competitive interactions with each other, e.g. through the secretion of toxic compounds or expression of effective contact-dependent inhibition mechanisms (Hibbing et al. 2010; Cornforth and Foster 2013; Ruhe et al. 2013; Ho et al. 2014; Russell et al. 2014; Stubbendieck and Straight 2016).

Cooperation and competition are well-defined concepts, based on studies with higher organisms. In past years, they were also applied to microbes. According to Hamilton (Hamilton 1964) we can distinguish between four types of social interactions, depending on the fitness effects on the actor and the recipient (Figure 1).

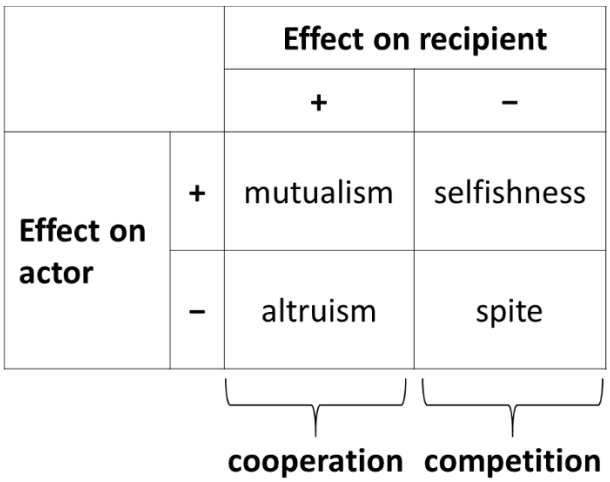


Figure 1: According to Hamilton (Hamilton 1964) *we can distinguish 4 types of social behavior. The actor and recipient engage in either cooperative or competitive behavior. A mutualistic behavior is beneficial to both participants, while altruism benefits only the recipient. A spiteful act decreases the fitness of both participants. The selfish behavior increases fitness of the actor but reduces fitness of the recipient* (West et al. 2007).

The four types of social interactions entail both cooperative and competitive behaviors. Mutualistic and altruistic acts are cooperative behaviors carried out by the actor to increase fitness of the recipient, while selfish and spiteful interaction are forms of competition that aim to suppress the recipient's fitness (Figure 1) (Hamilton 1964; West et al. 2007).

1.1.2. Cooperation is widespread among bacteria

Among bacteria, cooperation through mutualism and altruism is a wide spread phenomenon including the above mentioned examples QS, biofilm formation and iron uptake through siderophores. Cooperation is mediated largely by public goods that are secreted and shared among the population. Siderophores and extracellular proteases for example are well studied public goods cooperation systems (Griffin et al. 2004; Harrison et al. 2006; Dumas and Kümmerli 2012; Smalley et al. 2015). Natural selection, however, favors cooperative behavior only under certain conditions that can be described by Hamilton's rule: $rb > c$ (Hamilton 1964). It says that cooperation is only favored, when the costs of the cooperative act (c) are lower than the benefits for the recipient (b) weighted by the relatedness (r) between the actor and recipient. Hamilton's rule also explains why most cooperative behavior can be found among kin or closely related individuals. Among clonal populations of bacteria, where r=1, cooperation should thus be especially strong. Cooperative interactions among kin increase the inclusive fitness of the actor by increasing its own proliferation (direct fitness effects) and increasing the proliferation of its kin cells (indirect fitness effects) (Hamilton 1964; West et al. 2006, 2007).

1.1.3. Competition occurs between but also within species

Bacteria do not only cooperate, but can also engage in competitive interactions with one another. Competition can occur between members of the same strain, but also between different species. Intra-specific competition can often directly emerge from cooperation in the form of social cheats that exploit the cooperative effort. Cheats are mutants that stopped producing costly public goods but still free ride on the compounds produced by others (West et al. 2006; Harrison and Buckling 2009; Asfahl and Schuster 2017). At the inter-specific level, competition predominantly occurs for space and nutrients. Selfish behavior and spite decrease the fitness of the competitor to maximize the fitness of the actor, or the actor's indirect fitness, respectively. (Hamilton 1963, 1964; Hawlena et al. 2010). Spite in bacterial communities is common and often carried out by the production of metabolically costly bacteriocins. *Escherichia coli* releases its bacteriocins through cell lysis, which is the ultimate cost for the producing cells (Riley and Wertz 2002).

Competitive behavior can be further distinguished into indirect exploitative resource competition and direct interference competition, depending on the means that are applied to suppress the opponent's fitness. In indirect resource competition one competitor depletes the resources of another, while direct interference competition is characterized by the production of compounds that directly harm the competitor (Cornforth and Foster 2013). Highly related bacteria are likely to use the same resources. Thus, we would expect high levels of resource competition and little interference competition. One factor that further contributes to this relationship is that bacteria usually express immunity-conveying mechanisms to prevent self-intoxication, which also render these bacteria insensitive to attacks by clone mates (Russell et al. 2011; Hayes et al. 2014; Jamet and Nassif 2015). Nevertheless, resource and interference competition can be applied simultaneously to maximize the fitness gain for the actor over the competitor. Nutrients like iron or specific carbon sources are commonly available and are taken up by more than just one bacterial species. For example, species can engage in competition for iron by secreting specific siderophores, whereby the common iron pool is mostly scavenged by the siderophore with the highest iron binding capacity (Weaver and Kolter 2004; Joshi et al. 2006; Harrison et al. 2008; Traxler et al. 2013; Schiessl et al. 2016; Niehus et al. 2017). Simultaneously bacteria could secrete toxic compounds that directly harm their competitors and reduce their fitness in the competition. Thus, bacterial competition is a complex interplay of many traits.

The examples above show that bacteria have multiple possibilities to counteract competition. Less well known however is, if bacteria can adjust their responses to match different levels of competition. Such phenotypic plasticity is well known from animals that adjust their aggressive behavior to the prevailing level of competition. The question is if bacteria can express a competitive phenotype, only when it is

beneficial to outcompete other microbes. It has been shown that microbes can change their phenotype in response to inter-specific competition i.e. by increasing biofilm formation or siderophore secretion (McKenney et al. 1995; Weaver and Kolter 2004; Harrison et al. 2008; Oliveira et al. 2015). However, it is not known if these phenotypes can be further regulated in response to different levels of competition.

If bacteria are able to express a plastic phenotype to modulate their response to different competition levels, it requires mechanisms for competition sensing. Cornforth and Foster propose two main mechanisms. First, bacteria detect environmental changes, like resource stress, oxidative or pH stress, and subsequently activate general stress responses. Although environmental fluctuations can be caused by general environmental factors, they can also be the result of normal bacterial metabolism and thus convey the presence of other microbes in the habitat. Second, competition can be sensed directly through secreted metabolites and enzymes derived from competing species or through cell-cell contact (Cornforth and Foster 2013). It could be possible, that the two sensing mechanisms induce different kinds of responses in the recipient like resource interference competition. Furthermore, bacteria could also express a phenotype that helps to avoid or escape from competing species, e.g. by forming biofilms, or increasing flagellar movement.

1.1.4. Maintaining biodiversity in microbial communities

One key interest in ecology is to explain how biodiversity can be stabilized given the prevalence of strong competition within and between species. In other terms, there is great interest to understand the mechanisms that prevent stronger species to outcompete and replace their weaker counterparts.

The high biodiversity in multispecies bacterial communities can potentially be explained by several ecological theories. One way to stabilize biodiversity is through spatial structure in natural habitats like soil or host tissue. Bacteria itself can also form highly structured biofilms. In structured environments, weaker community members can find refuge from dominant ones to prevent being outcompeted. Spatial structure influences the bacterial community mainly through limiting diffusion of chemical compounds and distribution of bacteria. This leads to shorter ranges, where toxins are active, which prevents sensitive cells from being damaged. Furthermore, local relatedness is increased, thus reducing between species competition (Kerr et al. 2002; Kim et al. 2008; Vos et al. 2013), but simultaneously increasing competition between clone mates (Kümmerli et al. 2009a). Besides affecting competitive interactions, spatial structure also stabilizes cooperation by keeping cooperators close together and restricting the diffusion of public goods away from the producers. Thus, access of non-

producing cheats to diffusible public goods is limited (Allen et al. 2013; Bruger and Waters 2015; Özkaya et al. 2017).

Niche construction is another mechanism that contributes to the maintenance of biodiversity, where one species increases the fitness of another one in an otherwise hostile environment, which enables the coexistence of the two species. This can happen for example by the secretion of metabolic byproducts, that feed other species (Poltak and Cooper 2011; Seth and Taga 2014), or through the degradation of toxic compounds like antibiotics, which also benefits other species (Vega and Gore 2014; Kelsic et al. 2015; McNally and Brown 2015; Meredith et al. 2015). Further, providing shelter from harsh environments, i.e. through biofilm formation, is a form of niche construction (McNally and Brown 2015).

Non-transitivity is a special case, where several bacterial strains compete for the same resource yet still can coexist, since there is no overall dominant species that could outcompete all other strains. One famous example describes a community of toxin secreting (T), resistant (R) and susceptible (S) *Escherichia coli* strains. S outgrows R because it saves the costs of resistance. R outgrows T, because it saves the costs of toxin production, whereas T needs to invest in the production of the toxin and resistance mechanism. T outcompetes S because of killing. Thus, the three strains can coexist in a rock-paper-scissors like relationship (Kerr et al. 2002; Kirkup and Riley 2004). Inglis et al. demonstrated a similar system among *Pseudomonas aeruginosa* strains, which secrete two different types of pyoverdine, a small peptide siderophore that binds environmental iron. One strain secretes type 1 pyoverdine (P1), another strain is a defector (D1) that can free-ride on the type 1 pyoverdine, and the third strain of the community secretes an independent type 2 pyoverdine (P2) that cannot be taken up by the other two strains. Here, all three strains coexist in a non-transitive manner, although they outcompete each other in 2-way competitions. P1 outcompetes P2 due to its more efficient siderophore, P2 outcompetes D1, because D1 cannot free ride on the siderophore of P2, and D1 outcompetes P1 since it exploits the cooperative secretion of siderophores (Inglis et al. 2016).

In conclusion, type and intensity of social interactions within and between species are determined by the environment. Together they influence the biodiversity in multispecies bacterial communities.

1.2. The importance of iron in cooperation and competition

In my thesis, I focus on the competition for iron. Iron is an essential nutrient for almost every living organism, yet its availability in nature is limited. Therefore, iron competition is a model system to study competition for limited resources within and between species. In this section, I first explain why iron is an essential nutrient. I will then illustrate how the two model organisms I used in my thesis, *Pseudomonas aeruginosa* (PA) and *Burkholderia cenocepacia* (BC), cope with iron limitation.

1.2.1. Biological significance of iron

Iron is the fourth most abundant element on earth and essential for nearly all forms of live (Crichton 2001; Fox et al. 2015). It exists in two stable oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron under different environmental conditions. Because of these redox properties, iron is used as a cofactor for many proteins, and thus plays a key role in crucial cellular pathways, like the electron transfer, oxygen transport, DNA synthesis or carbon metabolism (Crichton 2001; Fox et al. 2015). Despite its high abundance, iron is usually not freely available in nature. The readily soluble ferrous Fe^{2+} ion occurs only in anaerobic conditions or at very low pH values. Under high oxygen concentrations, the ferric Fe^{3+} ion is predominant. However, its water solubility is extremely low (Crichton 2001). Additionally in mammalian hosts, iron is bound by proteins to render it even less available for microbial pathogens (Latunde-Dada 2009). Therefore, bacteria evolved mechanisms to actively scavenge ferric iron from their environments (Chu et al. 2010). One way to do so, is by secreting siderophores, small highly iron affine peptides (Latunde-Dada 2009; Saha et al. 2013). Siderophores are a widespread method for iron uptake that can also be found in fungi and plants (Ahmed and Holmström 2014).

1.2.2. Iron uptake in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (PA) is a gram-negative ubiquitous bacterium that is well known for being an opportunistic human pathogen with resistance mechanisms against a large range of commonly used antibiotics. The World Health Organization classified bacterial pathogens into different categories to guide research and development of new antibiotics treatments. PA was classified into the highest priority of this list(World Health Organization 2017). PA carries a large genome with 6.3 million base pairs that form 5570 open reading frames. The high number of genes reflects that PA is a generalist, when it comes to occupying habitats and using nutrients (Stover et al. 2000). It is one of the best studied bacterial species, with regard to cooperation and competition (Dunny et al. 2008; Harrison et al. 2008; Harrison and Buckling 2009; Kümmerli and Brown 2010; Wilder et al. 2011; Tashiro et al.

2013). Therefore, I use it as one model organism in my thesis. PA lives in versatile environments ranging from marine over soil habitats to human hosts (Stover et al. 2000; Cao et al. 2001). To overcome the low bioavailability in these habitats PA employs several iron scavenging mechanisms. The uptake of heme, an iron-containing cofactor for enzymes important in electron and oxygen transport, is one way for PA to scavenge iron. Another possibility is to reduce ferric iron extracellularly to ferrous iron by secreting reducing agents like phenazines. The soluble ferrous iron can then be taken up by the Feo-system in the PA cell envelop (Cornelis and Dingemans 2013). The best-studied iron uptake systems in PA, however, are its siderophores. Like other bacteria, PA uses two complementary siderophores for iron acquisition, pyoverdine and pyochelin (pch) (Cornelis 2010; Cornelis and Dingemans 2013; Tyrrell and Callaghan 2016). It was suggested that PA produces mainly the more efficient, yet more costly, pyoverdine when iron availability is low, but switches to the cheaper less efficient pyochelin (pch) when iron availability increases (Brandel et al. 2012; Dumas et al. 2013). Both siderophores are produced by several enzymes in a non-ribosomal peptide synthesis (Visca et al. 2007; Cornelis 2010). However, more enzymes are involved in the pyoverdine synthesis, which makes this peptide metabolically more costly to produce than pch (Cornelis 2010). Due to its high iron affinity, pyoverdine can displace iron directly from host proteins that usually withhold iron from bacteria as part of the host defense against pathogens. Thus pyoverdine is an important virulence factor (Wolz et al. 1994; Xiao and Kisaalita 1997; Chu et al. 2010; Granato et al. 2016).

1.2.2.1. Regulation of pyoverdine and pyochelin synthesis

Because pyoverdine is so costly to produce, the pyoverdine synthesis is tightly regulated by positive and negative feedback loops via the transcriptional regulators PvdS and ferric uptake regulator (Fur), respectively (Cornelis 2010). PvdS is bound by its antisigma factor FpvR to the cytosolic side of the inner cell membrane. FpvR itself forms a membrane spanning complex together with the specific ferri-pyoverdine receptor FpvA (Lamont et al. 2002). When ferri-pyoverdine binds to FpvA, it is transported into the periplasm, where ferric iron is reduced and thus removed from the pyoverdine molecule. Ferrous iron is transported into the cell and the pyoverdine molecule can be subsequently recycled to scavenge more iron (Imperi et al. 2009). The binding of ferri-pyoverdine to its receptor initiates a signaling cascade through FpvR, upon which PvdS is released into the cytosol. It can now bind to the promoters of pyoverdine synthesis genes and other virulence genes and activate their transcription (Lamont et al. 2002; Reinhart and Oglesby-Sherrouse 2016). To balance this reaction and to prevent an overproduction of pyoverdine, its synthesis is negatively controlled by the master regulator Fur (Ochsner et al. 1995; Cornelis 2010). This regulation is highly sensitive to the cellular iron concentration. When the intracellular iron concentration rises, it forms a complex with the otherwise

inactive Fur. This complex binds to Fur-boxes close to the promoter sites of target genes and thus actively blocks their expression. One of those target genes is *pvdS*, which encodes the sigma factor that regulates pyoverdine synthesis (Leoni et al. 2000; Cornelis et al. 2009; Cornelis 2010; Reinhart and Oglesby-Sherrouse 2016). Fur also blocks the transcription of pch synthesis genes and pchR, encoding a transcription regulator, under iron repletion. When iron is scarce, Fur is removed from the Fur boxes of the pch synthesis genes and the *pchR* gene. Thus, the expression of these genes is activated. Intracellular pch as effector together with PchR further stimulate the transcription of pch synthesis genes (Poole and McKay 2003; Michel et al. 2005).

1.2.2.2. Pyoverdine and pyochelin are public goods

Pyoverdine is a model trait to study cooperation in public goods production (Buckling et al. 2007; Harrison and Buckling 2009; Kümmerli et al. 2015). Taking up pyoverdine as an iron scavenger brings a fitness benefit to the recipient. However, building up the enzymatic production machinery for pyoverdine is very costly. Thus, social cheats that stop investing in the production, but keep the receptors for pyoverdine uptake, have a fitness advantage over the cooperators (Griffin et al. 2004; Harrison and Buckling 2009; Jiricny et al. 2010). The same interactions can be observed for PA's secondary siderophore pch (Ross-Gillespie et al. 2015). It has been shown, that pyoverdine non-producers readily evolve in laboratory cultures (Dumas and Kümmerli 2012; Kümmerli et al. 2015). But they are also commonly found in hosts infected with PA and in natural settings (De Vos et al. 2001; Andersen et al. 2015; Butaitė et al. 2017) indicating that cheating is a common phenomenon in nature.

1.2.3. Iron uptake in *Burkholderia cenocepacia*

The second model organism that I work with in my thesis is *Burkholderia cenocepacia* (BC). It belongs to the group of closely related *Burkholderia cepacia* complex (BCC) species that are best known as environmental species, which mostly colonize soil habitats and the plant rhizosphere (Coenye and Vandamme 2003; Vial et al. 2011; Eberl and Vandamme 2016).But BCC species are also important opportunistic human pathogens that cause serious infections in human patients. One well-studied example are BCC infections in lungs of cystic fibrosis patients (Drevinek and Mahenthiralingam 2010; Vial et al. 2011). Similar to PA, BC possesses several distinct iron scavenging systems. It can take up heme and ferritin as iron sources. The latter functions as an iron depot in many organisms (Whitby et al. 2006; Thomas 2007). BC further expresses three mechanisms to specifically take up ferric iron from its environment. One of them is the membrane-associated Ftr_{BCC}ABCD system, that shuffles ferric iron

across the cell membrane into the cytosol (Mathew et al. 2014). In addition, BC features two siderophore systems, ornibactin (orb) and pyochelin (pch). Ornibactin, as the primary siderophore of BC, binds ferric iron with a higher iron affinity than pch (Visser et al. 2004; Thomas 2007). The organization and regulation of the pch synthesis genes in BC is similar to the one in PA (Thomas 2007). Orb and pyoverdine also show some similarities. Both siderophores from BC and PA, respectively, are produced by non-ribosomal peptide synthesis, and they have a similar molecular structure (Agnoli et al. 2006b). The transcription of the orb synthesis genes is regulated by the transcription factor OrbS, which is controlled by Fur in response to cellular iron concentrations. However, OrbS cannot activate its own transcription, in contrast to the pyoverdine transcription factor PvdS (Stephan et al. 1993; Agnoli et al. 2006a).

Other as for PA, the siderophores of BC have not been investigated as a model for public goods. Therefore, it is still unknown if orb or pch non-producers evolve over time, and if these mutants can cheat on BC siderophore producers.

1.2.4. The role of siderophores in competition

Siderophores are usually seen in the light of cooperation and the public goods dilemma, as they are secreted as public goods to scavenge iron from the environment. All community members with the respective receptor, including so called “cheats” that do not contribute to the production, can benefit from the cooperative effort (Griffin et al. 2004; Harrison and Buckling 2009; Jiricny et al. 2010). In recent years, however, it became evident that siderophores, apart from the intra-specific competition for public goods, also mediate competition between species. Competition for iron in natural settings is usually high, since the bioavailability of iron is low. Therefore, not only bacteria, but also fungi and plants, secrete siderophores to actively take iron up from the environment, and withhold it from competitors (Ahmed and Holmström 2014). Also in the context of pathogens, interspecific competition for iron should not be overlooked given the diverse interactions between pathogens and beneficial commensals (Kamada et al. 2013; Zhang and He 2015), and also the fact that many diseases are caused by polymicrobial infections (Peters et al. 2012; Murray et al. 2014).

Two different scenarios are conceivable, where siderophores mediate inter-specific competition. First, bacteria can take up xenosiderophores and thus deplete the public goods pool of other species. It is common among bacteria, to carry receptors for siderophores that they do not synthesize themselves (Cornelis 2010; Traxler et al. 2012; Galet et al. 2015; Harrington et al. 2015). This process is also called “siderophore piracy” (Traxler et al. 2012). PA is a bacterium that carries receptors for an especially large numbers of xenosiderophores, that can be useful in the various habitats it lives in (Cornelis 2010;

Cornelis and Dingemans 2013). Some bacteria even seem to rely completely on xenosiderophores for iron acquisition in specific environments. This idea is supported by the fact, that siderophores of unrelated species enable growth of otherwise unculturable bacteria under laboratory conditions (D'Onofrio et al. 2010; Traxler et al. 2012).

Secondly, siderophores can be used to privatize iron and increased siderophore secretion can help to outcompete competitors. (Niehus et al. 2017). In this case, siderophores function as public goods on the level of the clonal population, but as private goods on the level of the multispecies community (Joshi et al. 2008). A dominant species could bind iron before competing species can do so by secreting siderophores with higher iron affinity that cannot be taken up by other microorganisms and by increasing the amount of siderophores. This phenomenon has been observed in several experimental studies with different bacterial species (McKenney et al. 1995; Weaver and Kolter 2004; Joshi et al. 2006; Harrison et al. 2008; Traxler et al. 2013; Tyrrell et al. 2015). Here, siderophore secretion creates a locally iron limited environment around the producer under low and high iron availabilities alike, which deprives competitors of the essential nutrient and lowers their fitness (Schiessl et al. 2016). This mechanism could explain how rhizosphere bacteria fulfill their function as plant protectants and consequently as supporters for plant growth (Buyer and Leong 1986; Haas and Défago 2005; Beneduzi et al. 2012).

1.3. The *Pseudomonas aeruginosa – Burkholderia cenocepacia* model system

In my thesis, I focused on the competition within and between species for the limited, yet essential nutrient iron. I specifically wanted to know how cooperation to scavenge iron from the environment affects the competition between species. Cooperation and competition can change the overall fitness of bacteria in a community, and thus could influence the whole community composition. Further, I wanted to know how inter-specific competition changes the phenotypic response and the underlying regulation of gene expression of individual species. To address these problems, I used a model community consisting of the two gram-negative bacterial species PA and BC. It is important to understand the interplay between these two species, because PA and BC can cause fatal infections in immunocompromised patients, especially CF patients, and are particularly hard to treat due to multiple resistances against antibiotics (Lambiase et al. 2006; Harrison 2007; Schwab et al. 2014; Schaffer 2015; Moradali et al. 2017; Scoffone et al. 2017; Stefani S et al. 2017). There are several reasons for why PA and BC mixed communities are a good laboratory model system to address questions related to competition and co-existence. First, PA and BC do not only co-infect lungs of CF patients but can also coexist in other natural habitats, like soil (Coenye and Vandamme 2003; Weaver

and Kolter 2004; Harrison 2007; Fang et al. 2011; Aujoulat et al. 2012; Maravic et al. 2012; Suzuki et al. 2013; Hariprasad et al. 2014; Nair et al. 2015; Schaffer 2015). Second, the genomes of both species are fully sequenced, which simplifies genetic manipulations and gene expression studies (Stover et al. 2000; Carlier et al. 2014). Additionally, many traits of PA and BC, including the iron uptake systems, are well characterized (Thomas 2007; Drevinek and Mahenthiralingam 2010; Subramoni and Sokol 2012; Cornelis and Dingemans 2013; Fazli et al. 2014; Mathew et al. 2014; Moradali et al. 2017), and the pyoverdine production in PA is easily tractable due to autofluorescence of the secreted molecule (Kümmerli et al. 2009c; Julou et al. 2013; Weigert and Kümmerli 2017).

To prevail in a wide range of habitats, PA needs to compete against many different microbial species and therefore needed to evolve various competitive strategies. Indeed, it has been shown that PA is able to outcompete numerous other microbial species, including gram-negative and gram-positive bacteria, and fungi (Mashburn et al. 2005; Costello et al. 2014; Trejo-Hernández et al. 2014; Filkins et al. 2015; Bernier et al. 2016). Some of the competitive strategies that PA also employs against BC include mechanisms of resource and interference competition. Resource competition for iron between PA and BC can be mediated by PA's highly iron-affine siderophore pyoverdin (Weaver and Kolter 2004; Harrison et al. 2008). Furthermore, PA produces highly toxic compounds that can mediate interference competition like pyocyanine or hydrogen cyanide (Baron et al. 1989; Tashiro et al. 2013; Jayaseelan et al. 2014; Smalley et al. 2015; Bernier et al. 2016). The type-6-secretion system can be used by PA to directly inject toxic enzymes into the cells of its competitor BC (Tashiro et al. 2013; Sana et al. 2016). The outcome of inter-specific competition between PA and BC, however, is influenced by environmental conditions. The lifestyle of the bacteria (sessile or planktonic), or the oxygen saturation of the medium determine if PA and BC can coexist, or if PA outcompetes BC (Riedel et al. 2001; Bragonzi et al. 2012; Costello et al. 2014; Schwab et al. 2014).

2. Aims of this Thesis

Bacteria live in multispecies communities where competition for common resources is high within and between species. In my thesis, I focused on the competition for iron, as an essential nutrient, within and between species. Iron can be taken up by cooperatively secreted siderophores and this cooperation between individuals of one species can influence the competition with other species of the community. Here, I focused on the effect of these interactions on the coexistence of PA and BC in a model community. I further investigated how between-species competition for iron affects the gene expression and the phenotype of PA.

In my first project, I examine how interactions between PA siderphore producers and their isogenic cheating mutants affect the competition with BC. I hypothesize that intra-specific competition through cheats within PA can reduce inter-specific competition, and foster co-existence between a stronger (PA) and a weaker competitor (BC), and that this mechanism can stabilize biodiversity even in well mixed conditions. My reasoning is that cheating compromises the fitness of PA, so that the competitive pressure on BC is released and both species can coexist. I tested my hypothesis in iron limited and rich medium, as well as in media differing in the level of spatial structuring, parameters that change the relative fitness benefit of the PA cheatby rendering siderophores non-essential, or limiting access of the PA cheats to siderophores by decreasing the diffusion rate.

In my second project, I investigated the role of phenotypic plasticity on inter-specific bacterial competition. It is well established that higher organisms exhibit phenotypic plasticity in response to different levels of competition (Agrawal 2001; Boege 2010). It is unclear whether bacteria can do the same. While it was shown that bacteria can increase their siderophore production in response to inter-specific competition (McKenney et al. 1995; Weaver and Kolter 2004; Harrison et al. 2008), it is not known whether bacteria can regulate their siderophore production to match prevailing levels of competition, in a similar manner as animals plastically regulate their aggressive behavior. To answer this question, I grew PA in the presence of different BC cell densities and compared the pyoverdine production in the mixed cultures with production in respective PA monocultures. I wanted to know if PA can gradually adjust its pyoverdine production in response to different levels of competition by BC, or if it changes the pyoverdine production in a binary manner according to the presence or absence of BC as a competitor. I further investigated if the phenotypic plasticity in pyoverdine production has fitness consequences in PA. I hypothesize that fitness consequences should be highest, when the competition for the common resource iron is strongest, which is under low general iron availability and when the BC cell density is large.

In my third project I investigate if PA responds to diffusible cues from BC at the global gene expression level. I further wanted to know if PA's response to these danger cues is to activate a defense mode, or if it prepares to attack the inter-specific competitor. In recent years, PA has been investigated in the light of being a versatile and ubiquitous bacterium that colonizes many different habitats including diverse multi-species communities (Aujoulat et al. 2012). While several studies looked at single traits that contribute to PA's strong competitiveness against other microbial species, I here conduct a global study of PA's response to inter-specific competition. I used RNA sequencing, to find out which specific traits are activated by PA, when sensing diffusible cues from its competitor BC. Therefore, I grew PA in a mixture of fresh medium and BC spent culture supernatant, so that the PA could receive cues from BC only through diffusible signals, but not from direct cell-cell contact (Cornforth and Foster 2013; Westhoff et al. 2017). I analyzed the differential gene expression of PA relative to growth in its own supernatant or only fresh medium. I was specifically interested in the regulation of genes encoding traits that could facilitate social interactions, like secretion systems, secreted enzymes, the synthesis of secreted secondary metabolites and the planktonic (swimming) or sessile (biofilm formation) lifestyle. I investigated if PA's response to diffusible competition cues from BC is multifactorial or based on single gene sets. I further varied the iron availability of the medium to find out, if the transcriptional response to competition is influenced by the nutritional status of the environment.

3. First Project: Cheating fosters species co-existence in well-mixed bacterial communities

This research was originally published in “The International Society of Microbial Ecology (ISME) Journal”.

Leinweber, A. [1]**, R. Fredrik Inglis** [2]**, and R. Kümmerli** [1]. 2017. Cheating fosters species co-existence in well-mixed bacterial communities. ISME J. 11:1179–1188.

[1] Department of Plant and Microbial Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

[2] Department of Biology, Washington University in St. Louis, St. Louis, MO, USA

doi:10.1038/ismej.2016.195

The ISME Journal (2017), 1–10

www.nature.com/ismej

ORIGINAL ARTICLE

Cheating fosters species co-existence in well-mixed bacterial communities

Anne Leinweber[1], R Fredrik Inglis[2] and Rolf Kümmerli[1]
[1]*Department of Plant and Microbial Biology, University of Zurich, Zurich, Switzerland and* [2]*Department of Biology, Washington University in St. Louis, St. Louis, MO, USA*

Explaining the enormous biodiversity observed in bacterial communities is challenging because ecological theory predicts that competition between species occupying the same niche should lead to the exclusion of less competitive community members. Competitive exclusion should be particularly strong when species compete for a single limiting resource or live in unstructured habitats that offer no refuge for weaker competitors. Here, we describe the 'cheating effect', a form of intra-specific competition that can counterbalance between-species competition, thereby fostering biodiversity in unstructured habitats. Using experimental communities consisting of the strong competitor *Pseudomonas aeruginosa* (PA) and its weaker counterpart *Burkholderia cenocepacia* (BC), we show that co-existence is impossible when the two species compete for a single limiting resource, iron. However, when introducing a PA cheating mutant, which specifically exploits the iron-scavenging siderophores produced by the PA wild type, we found that biodiversity was preserved under well-mixed conditions where PA cheats could outcompete the PA wild type. Cheating fosters biodiversity in our system because it creates strong intra-specific competition, which equalizes fitness differences between PA and BC. Our study identifies cheating – typically considered a destructive element – as a constructive force in shaping biodiversity.
The ISME Journal advance online publication, 6 January 2017; doi:10.1038/ismej.2016.195

Introduction

Most habitats on earth are populated by diverse bacterial communities (Curtis *et al.*, 2002; Rusch *et al.*, 2007; The Human Microbiome Project, 2012; Rinke *et al.*, 2013; Sunagawa *et al.*, 2015). Explaining the evolution and maintenance of this enormous bacterial biodiversity is challenging because competition between species occupying the same niche should often result in the exclusion of competitively inferior community members (Hardin, 1960; Becker *et al.*, 2012; Foster and Bell, 2012). Ecological theory predicts that species can only co-exist if intra-specific competition exceeds inter-specific competition, a scenario that can apply if competing species differ in their resource use or are spatially separated from one another (Holt *et al.*, 1994; Chesson, 2000; Lankau, 2009; Barabás *et al.*, 2016). Previous experimental work has demonstrated that these two mechanisms indeed apply to microbial systems. In toxin-mediated interference competition, for instance, spatial structure can prevent competitive exclusion because it allows weaker community members to find refuge from their more competitive counterparts (Kerr *et al.*, 2002; Kim *et al.*, 2008; Vos *et al.*, 2013). Moreover, it has been shown that evolving bacterial populations rapidly diversify, which promotes niche differentiation and reduces inter-specific resource competition (Rosenzweig *et al.*, 1994; Rainey and Travisano, 1998; Brockhurst *et al.*, 2006).

Here, we propose a novel mechanism of how increased intra- vs inter-specific competition can foster species co-existence, even under conditions where bacterial species compete for a single limiting resource in an unstructured, well-mixed habitat. In particular, we argue that a common form of intra-specific competition in bacteria is social cheating, where bacteria, secreting a group-beneficial compound, are exploited by 'cheating' variants of the same species (Rainey and Rainey, 2003; Griffin *et al.*, 2004; Cordero *et al.*, 2012; Raymond *et al.*, 2012). Cheats are mutants that have lost the ability to produce the costly compound but still free ride on the compounds produced by others. Following the theoretical considerations and terminology of Chesson (2000) we predict that cheating has both equalizing and stabilizing fitness effects. If cheating occurs in the more competitive species then cheats equalize fitness differences between species, because they exclusively compromise the fitness of their own species. By doing so, they also increase intra-specific

Correspondence: R Kümmerli, Department of Plant and Microbial Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland.
E-mail: rolf.kuemmerli@uzh.ch
Received 8 July 2016; revised 28 October 2016; accepted 7 December 2016

competition, creating a stabilizing fitness effect. Here, we empirically test these predictions and examine whether within-species cheating is a strong enough force to foster species co-existence and biodiversity.

We tested these predictions by constructing experimental bacterial communities consisting of *Pseudomonas aeruginosa* (PA) and *Burkholderia cenocepacia* (BC), two opportunistic human pathogens. These two species are notoriously hard to maintain in planktonic laboratory co-cultures because PA is a very strong competitor and is able to quickly suppress BC (Bragonzi *et al.*, 2012; Costello *et al.*, 2014). Although previous work examined the interactions between these two species because they can simultaneously be isolated from soils and infections (Weaver and Kolter, 2004; Lambiase *et al.*, 2006), we here use them as a model system to test fundamental ecological theory. To match conditions where the competitive exclusion principle applies (Hardin, 1960), we created an environment where the two species compete for a single limiting resource – iron: a key essential nutrient, limiting bacterial growth both in natural environments (Hersman *et al.*, 2000) and within hosts (Miethke and Marahiel, 2007). In order to scavenge iron, both species rely on the secretion of small peptide derivates (pyoverdine for PA and ornibactin for BC), called siderophores (Miethke and Marahiel, 2007; Tyrrell and Callaghan, 2015). Importantly, pyoverdine production in PA is a cooperative trait as secreted molecules can be shared as public goods within the *Pseudomonas* population (Griffin *et al.*, 2004). Pyoverdine sharing selects for cheating mutants, which exploit the pyoverdine produced by others without contributing to it themselves (Harrison *et al.*, 2008; Dumas and Kümmerli, 2012). Thus, to examine how cheating affects biodiversity, we introduced a cheating variant of PA into our community, which is unable to produce pyoverdine but possesses the receptor for its uptake.

We studied the competitive relationships and evolutionary dynamics between the PA wild type, the PA cheat and BC in pairwise combinations and in three-strain communities across a gradient of spatial structures, ranging from well-mixed (that is, low spatial structure) to viscous medium (that is, increased spatial structure). Consistent with the competitive exclusion principle, we found that the PA wild type completely displaced BC regardless of spatial structure. This dynamic changed when adding a PA cheat to the community, where, under low spatial structure, the PA cheat efficiently exploited the pyoverdine of the PA wild type, thereby weakening its competitive strength and fostering species co-existence. Conversely, increased spatial structure impeded biodiversity, as it prevented PA cheats from outcompeting the PA wild type, such that the latter could drive BC to extinction. Taken together, our experiments identify within-species cheating as a novel mechanism, which can promote biodiversity in habitats with low spatial structure.

Materials and methods

Bacterial strains, media and growth conditions

We used the PA strain PAO1 (ATCC 15692) as the superior competitor and the BC strain H111 (LMG 23991), an isolate from a cystic fibrosis patient (Gotschlich *et al.*, 2001), as the inferior competitor in our eco-system. The former produces pyoverdine and pyochelin, the primary and secondary siderophores of PA, respectively. H111 produces the primary siderophore ornibactin, but, as the PA wild type, it has also the ability to produce pyochelin as a secondary siderophore. Although it remains unknown whether this siderophore can be exchanged between species, it has been shown that under strong iron limitation, as imposed in our experiments, the production of pyochelin is negligible in both species (Dumas *et al.*, 2013; Tyrrell *et al.*, 2015). To rule out any pleiotropic effects associated with pyochelin production, we used the PA siderophore knock-out strain PAO1Δ*pvdD*Δ*pchEF* as the PA cheat. This strain can neither produce pyoverdine nor pyochelin. While this strain possesses the receptor to take up pyoverdine, it cannot use ornibactin from BC. To be able to distinguish strains in mixed competitions, we used variants of the PA wild type and the PA cheat that constitutively expressed eGfp and mCherry, respectively (chromosomal insertions *att*Tn7::Ptac-*egfp* and *att*Tn7::Ptac-*mcherry*). H111 did not carry a fluorescent marker. This strain was easily distinguishable from PA based on the absence of a fluorescent signal and its small dot-like colony morphology.

Overnight cultures were grown in lysogeny broth (LB). We induced strongly iron-limiting growth conditions by using casamino acids (CAA) medium (per 1 l: 5 g casamino acids; 1.18 g $K_2HPO_4{\cdot}3H_2O$; 0.25 g $MgSO_4{\cdot}7H_2O$) supplemented with 20 mM $NaHCO_3$, 25 mM HEPES and 100 μg ml^{-1} of the natural iron chelator human apo-transferrin. For control experiments in iron-replete medium, we omitted transferrin from the above recipe to create conditions where siderophores are not required for growth. We further manipulated the spatial structure of the environment by growing bacteria either in: (a) liquid shaken (170 r.p.m.) medium reflecting a relatively unstructured environment; (b) liquid static medium representing an environment with intermediate spatial structure; or (c) viscous static medium containing 0.1% agar reflecting an environment with relatively high spatial structure. All experiments were conducted at 37 °C. All chemicals were purchased from Sigma-Aldrich, Buchs, Switzerland.

Growth of PA and BC in iron-limited CAA medium

To quantify the growth capacities of our community members in the iron-limited media used, we estimated their number of doublings in monocultures in 18 replicates each, across 24 h. Therefore, overnight cultures were washed twice with 0.8% NaCl solution

and adjusted to $OD_{600} = 1$. Bacteria were then diluted and inoculated in 1.5 ml of CAA medium with supplements in 24-well plates to a starting OD_{600} of 1×10^{-4}. Plates were subsequently incubated for 24 h. We measured cell density at the beginning and at the end of the experiment by serially diluting cultures in 0.8% NaCl and plating aliquots on LB agar plates supplemented with 20 μM $FeCl_3$. These plates were incubated overnight and left at room temperature for another 24 h to allow bacterial colonies to fully mature. The colony-forming units (CFU) were then counted. The number of doublings was calculated as Doublings = $(\ln(x_t/x_0))/\ln(2)$, where x_0 and x_t are the initial and the final CFUs, respectively (Harrison *et al.*, 2008).

Competition experiments

To assess the competitive relationship between two strains, we washed overnight monocultures twice with 0.8% NaCl solution, adjusted them to $OD_{600} = 1$ and mixed the strains at specific ratios (see below for details). We inoculated mixes at a starting OD_{600} of 1×10^{-4} into 1.5 ml of iron-limited CAA medium on 24-well plates, and incubated the plates for 24 h. Before and after competition, we counted the CFUs of both competing strains by serially diluting cultures in 0.8% NaCl and plating competitions individually on LB agar plates supplemented with 20 μM $FeCl_3$. The plates were incubated overnight and left at room temperature for another 24 h to allow fluorescent markers to fully mature. We used a fluorescent imaging device (Lumenera Infinity 3 camera connected to a dark chamber) to differentiate between fluorescently tagged and untagged colonies. We then calculated the relative fitness (v) of the focal strain as $v = [a_t \times (1 - a_0)]/[a_0 \times (1 - a_t)]$, where a_0 and a_t are the initial and final frequency of the focal strain, respectively (Ross-Gillespie *et al.*, 2007). We ln-transformed all fitness values in order to obtain normally distributed residuals. Values of $\ln(v) > 0$ or $\ln(v) < 0$ indicate whether the frequency of the focal strain increased or decreased relative to its competitor, respectively.

In a first set of competition assays, we checked for fitness effects of the fluorescent markers mCherry and eGfp. We competed the untagged PA wild type against both the mCherry and eGfp tagged PA wild type (eight replicates per competition). We found that the constitutive expression of *egfp*, but not *mcherry*, was associated with a slight but significant fitness cost (one-sample *t*-test testing whether the relative fitness differs from zero: for PAO1 *egfp*, $\ln(v) = -0.358 \pm 0.13$ (mean ± 95% CI), $t_7 = -6.58$, $P = 0.0003$; for PAO1 *mcherry*, $\ln(v) = -0.027 \pm 0.21$, $t_7 = -0.31$, $P = 0.766$). To ensure that this marker effect does not alter the competitive abilities of PA wild type and BC, we decided to tag the PA wild type (the strong competitor in our community) with eGfp, whereas BC remained untagged. Indeed, it turned out that even with the eGfp handicap, the PA wild type was able to displace BC (Figure 2a).

We then performed competitions between all pairwise combinations of our strains (PA wild type vs BC, PA cheat vs BC, and PA wild type vs PA cheat) using three different starting ratios (1:9, 1:1, 9:1). We varied the starting frequencies to examine the robustness of competitive outcomes across a range of mixing ratios. We further manipulated the spatial structure of the environment along a gradient from unstructured to highly structured according to the procedure described above. The minimum level of replication was 12 (for low and intermediate spatial structures) and 7 (for high spatial structure). Note that in competitions between the PA wild type and BC, CFU counts for BC often equalled zero (that is, BC frequency was below the detection limit after competitions). In order to estimate the relative fitness of BC, we substituted CFU = 0 by CFU = 0.1.

Finally, to verify that it was indeed iron limitation that caused the observed competitive interactions in our community, we performed a control experiment (in 9-fold replication), where we competed the strains in a relatively iron-rich environment (CAA without transferrin) where siderophores are less important for iron scavenging.

Evolutionary dynamics in experimental communities

We followed the evolutionary strain dynamics in our three-strain community (PA wild type vs PA cheat vs BC, 6 replicates) and in all combinations of 2-strain communities (four replicates each) over 6 days in iron-limited medium both under unstructured (shaken liquid) and structured (static viscous) conditions. As an additional control we also grew the three-strain community in unstructured iron-rich media where siderophores are unimportant for iron-uptake. In total, we had 42 evolving communities. We transferred 10% of each community to fresh medium daily, except for the community BC vs PA cheat, which grew poorly. For this combination, we transferred 25% of the culture to prevent over-dilution and subsequent population extinction. Following each daily transfer, we assessed the strain proportions in each community using the procedure described above.

Statistical analysis

We used linear models (LM) for statistical tests, and false discovery rate tests were applied for *post hoc* multiple pairwise comparisons (Benjamini and Hochberg, 1995). In cases where data distribution did not entirely meet the assumptions of linear models, we performed non-parametric Mann–Whitney tests and one-sample Wilcoxon signed rank tests to test the robustness of the parametric analyses. All statistical analysis was carried out in R 3.1.2 (R Development Core Team, 2015).

Results

The PA wild type outgrows BC and the PA cheat in monoculture

Our monoculture experiments show that the PA wild type divided significantly faster than BC under

iron-limited conditions (LM: $t_{51} = 22.72$, $P < 0.0001$). Based on the huge fitness difference observed, we expect the PA wild type to outcompete BC in direct competition. In contrast, BC and the PA cheat did not significantly differ in their number of doublings over 24 h (LM: $t_{51} = 0.40$, $P = 0.694$; Figure 1). This suggests that cheats could have an equalizing fitness effect in mixed communities, as they drag down the overall growth potential of PA relative to BC. Moreover, because the PA wild type and the PA cheat only differ in their ability to produce siderophores, our data suggest that fitness differences between species are due to differences in siderophore efficiency: pyoverdine from PA is presumably the more efficient siderophore than ornibactin from BC.

PA wild type displaces BC in mixed cultures

As expected from the competitive exclusion principle (Hardin, 1960), our data show that co-existence between BC and the PA wild type is not possible in iron-limited medium. PA consistently outcompeted BC, regardless of the spatial structure of the medium (Figure 2a) and the starting frequency of BC (Supplementary Figure 1; one-sample *t*-test on pooled relative fitness values across all conditions: $t_{98} = -32.64$, $P < 0.0001$). In fact, BC was below the detection limit (mean detection limit = 1.9%) in 75.8% of all competitions. BC persistence was typically confined to replicates where BC starting frequency was 90%, but even for those cases BC counts were low (ranging between 1 and 8 CFU, average frequency 2.9%, Supplementary Figure 2 and Supplementary Table 1). This shows that PA is a very strong competitor that can drastically suppress BC within 24 h, precluding co-existence of both species.

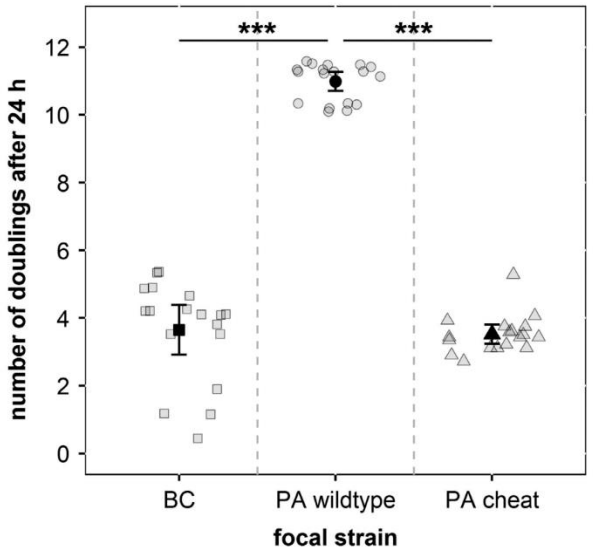


Figure 1 PA wild type grows significantly better than BC and the PA cheat in iron-limited medium (*** $P < 0.001$). This indicates that the PA cheats have an equalizing fitness effect in mixed communities, as they drag down the overall growth potential of PA relative to BC. As the PA wild type and the PA cheat are isogenic except for genes mediating siderophore production, these results indicate that pyoverdine is the most efficient iron-uptake system in this environment. Bacteria were grown as monocultures (18 replicates) in iron-limited medium for 24 h at 37 °C. Dark and faint symbols represent mean (±95% CI) and individual-replicate number of doublings, respectively.

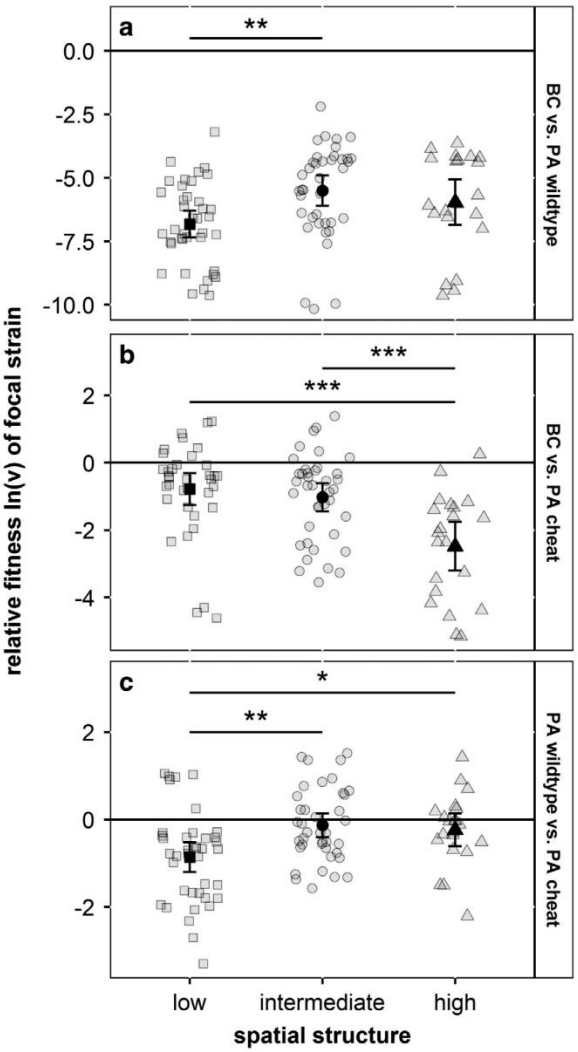


Figure 2 Relative fitness ln(v) in pairwise competitions between BC, PA wild type and PA cheat in iron-limited CAA medium across three different spatial structures. BC is outcompeted by the PA wild type at all spatial structures (**a**). The PA cheat is a weaker competitor than the PA wild type, but still significantly outcompetes BC at all spatial structures (**b**). The PA wild type is outcompeted by the PA cheat only under low spatial structure (**c**). The relative fitness ln(v) of the focal strain (the first strain in the panel headers) is shown for three levels of spatial structure (square = low, circle = intermediate, triangle = high), and was calculated after 24 h of competition. The values of $\ln(v) < 0$, $\ln(v) > 0$, or $\ln(v) = 0$, indicate whether the focal strain respectively lost, won, or performed equally well in competition with its opponent. Gray symbols represent individual data points, whereas black symbols indicate means ± 95% CI. Asterisks indicate significant differences between treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

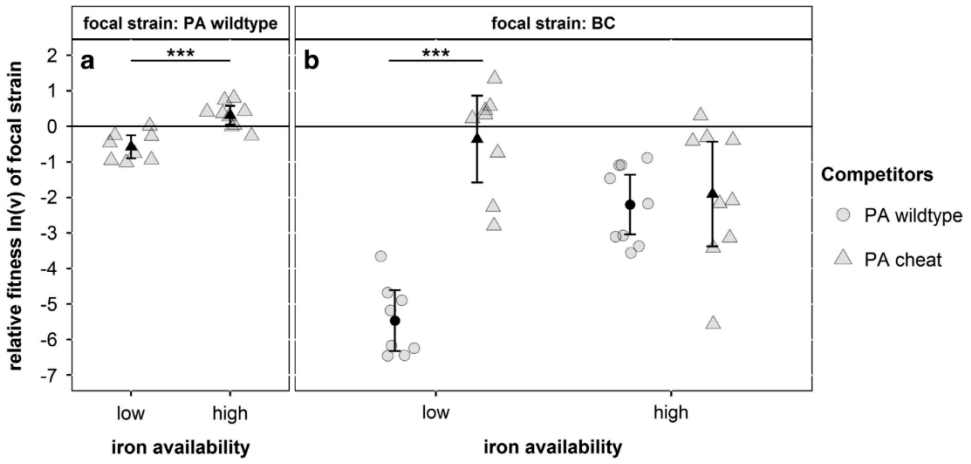


Figure 3 Iron availability alters the competitive relationships between community members. The PA wild type (PA wt) is only outcompeted by the PA cheat in low iron availability, but not when iron is more readily available (**a**). Under low iron availability, BC performs significantly better against the PA cheat than against the PA wild type (**b**). This effect disappears in media with increased iron availability, where the PA wild type and the PA cheat both outcompeted BC to an intermediate but equal extent (**b**). The values of ln(v) indicate whether the focal strain lost (ln(v)<0), won (ln(v)>0), or performed equally well (ln(v)$=0$) in competition with its opponent. Gray symbols represent individual data points (high iron availability: 9 replicates; low iron availability: 8 replicates), whereas black symbols indicate means ± 95% CI. Asterisks indicate significant differences between treatments (***$P<0.001$).

PA cheats change the competition dynamics

The PA cheat was significantly weaker in competition against BC than the PA wild type (LM: $F_{1,189}=447.9$, $P<0.0001$; Figure 2a vs b), showing the importance of siderophores in the competition between PA and BC. Although the PA cheat significantly outcompeted BC under all spatial structures (Figure 2b; one-sample t-tests for low spatial structure: $t_{95}=-3.34$, $P=0.002$; for intermediate spatial structure: $t_{95}=-5.02$, $P<0.0001$; for high spatial structure: $t_{95}=-7.18$, $P<0.0001$), there were significant differences between conditions. BC performed best in the medium with low spatial structure (low vs intermediate spatial structure, LM: $t_{93}=-3.86$, $P=0.0003$; and low vs high spatial structure, LM: $t_{93}=-4.45$, $P<0.0001$).

Spatial structure also significantly affected the relative fitness of the PA cheat in competition against the PA wild type (Figure 2c). In particular, the PA cheat was only able to significantly outgrow the PA wild type in low spatial structure (one-sample t-test: $t_{38}=-5.12$, $P<0.0001$), whereas both strains were equally fit in intermediate ($t_{38}=-0.96$, $P=0.34$) and high spatial structure ($t_{20}=-1.31$, $P=0.20$). These results show that siderophore cheats can only invade a population of cooperating siderophore producers in the absence of any significant spatial structure (confirming previous findings, Kümmerli *et al.*, 2009). This finding further demonstrates that the PA cheats' capacity to increase intra-specific competition (that is, exerting a stabilizing effect (Chesson, 2000)) is highest with low spatial structure.

Iron limitation is required for PA cheats to alter competitive dynamics

Control experiments revealed that it is indeed the 'cheating effect' – the PA cheat outcompetes the PA wild type, but is weaker in competition against BC – that affects the competitive dynamics in our iron-limited communities (Figure 3). When repeating all pairwise competitions in a medium where iron is more readily available and siderophores are no longer essential for iron scavenging, the cheating effect disappeared. Specifically, the relative fitness advantage the PA cheats enjoyed under low iron availability (one-sample t-test: $t_7=-4.19$, $P=0.0041$) vanished under increased iron availability (Figure 3a). Moreover, our previous observation that BC experiences higher relative fitness in competition against the PA cheat than against the PA wild type was only apparent with low iron availability (LM: $F_{1,14}=65.43$, $P<0.0001$), but not with high iron availability (LM: $F_{1,16}=0.162$, $P=0.693$; Figure 3b). These findings show that both the equalizing and stabilizing fitness effects only prevail under low iron availability. The two effects disappear under increased iron availability because the PA wild type and the PA cheat become phenotypically similar (that is, reduced pyoverdine expression, Supplementary Figure 3) and therefore suppress BC to the same extent.

Evolutionary dynamics in two-strain communities

Our six-day evolution experiments confirmed that BC was drastically suppressed by the PA wild type regardless of the spatial structure (Figures 4a+b; mean detection limit of BC = 0.27%, Supplementary Table 2). The dynamics significantly changed when replacing the PA wild type with the PA cheat. Here, the strain frequency fluctuated, under both low and high spatial structure, first in favor of the PA cheat and then, after the second day, in favor of BC (Figures 4c+d). The switch in the competitive strength between the two strains could be explained by phenotypic or genetic adaptations of BC to the

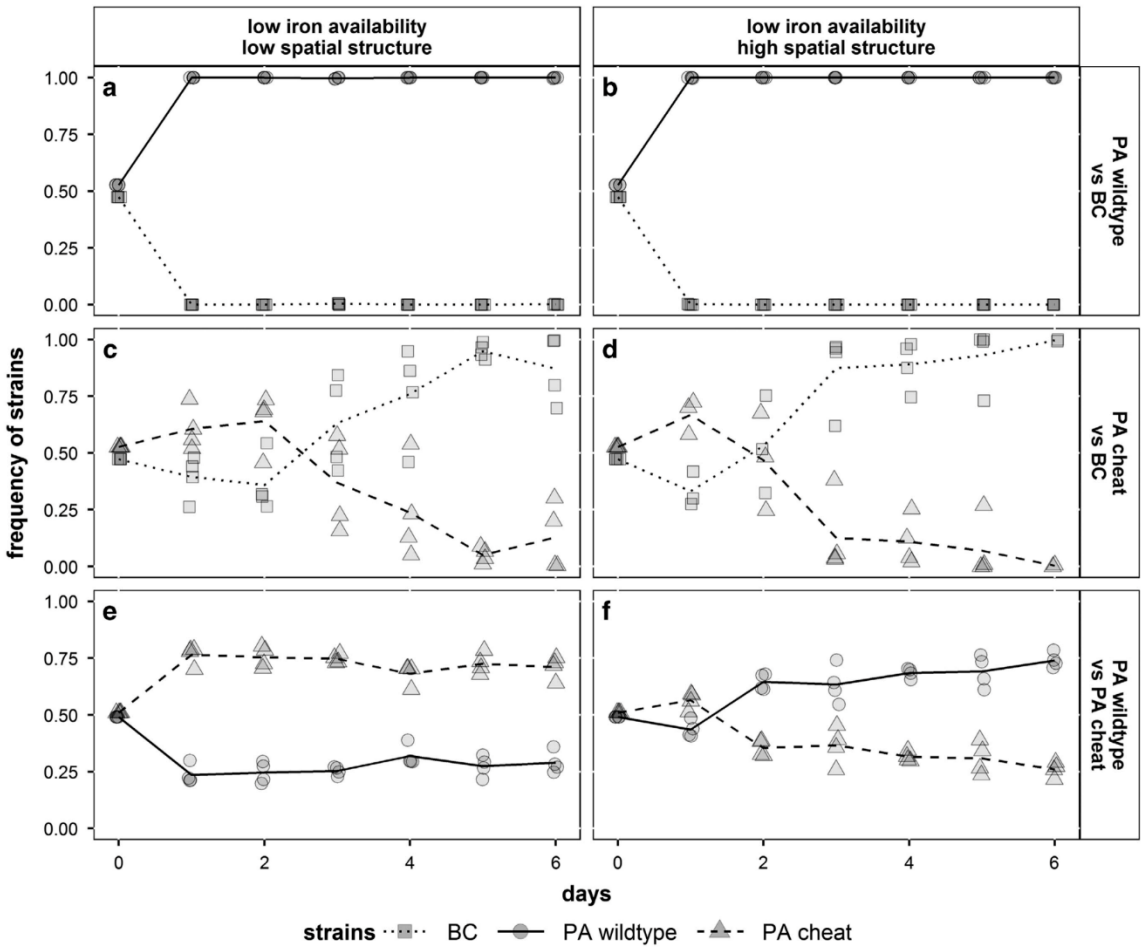


Figure 4 Evolutionary dynamics in two-strain communities with BC (squares, dotted line), PA wild type (circles, solid line) and PA cheat (triangles, dashed line) in low and highly structured iron-limited CAA medium. Co-existence of BC and PA wild type is not possible, neither in unstructured (**a**) nor structured medium (**b**). BC becomes the dominant strain in competition with the PA cheat after 2 or 3 days regardless of spatial structure (**c**, **d**). The PA cheat outcompetes the PA wild type only in medium with low spatial structure (**e**) but not in highly structured medium (**f**). Symbols depict individual data points (from four replicates), whereas lines represent mean strain frequencies.

presence of the PA cheat. Alternatively, it could be that PA cheats first benefited from internal iron stocks acquired during the pre-experimental growth in rich LB medium, which then became depleted during the course of the experiment. Finally, spatial structure strongly affected the long-term dynamics between the PA wild type and the PA cheat (Figures 4e+f). In line with our 24-h competition assays, we found that the PA cheat significantly outcompeted the PA wild type under low spatial structure, but was outcompeted itself by the PA wild type in the structured environment.

Evolutionary dynamics in three-strain communities

The two-strain evolutionary experiments above demonstrate that the PA cheat is a weak competitor against BC, but can itself significantly reduce the fitness of the PA wild type under low spatial structure and in iron-limited medium. Our experiments with three-strain communities demonstrate that this cheating effect was indeed sufficient to foster stable co-existence of BC, the PA wild type and the PA cheat over several days maintaining BC at low frequencies in the community (Figure 5a). Conversely, diversity could neither be sustained under high spatial structure (Figure 5b) nor under increased iron availability (Figure 5c; mean detection limit of BC on day 6 across all three conditions = 0.19%, Supplementary Table 2).

Discussion

Ecological theory predicts that species can locally co-exist if intra-specific competition exceeds inter-specific competition (Chesson, 2000; Lankau, 2009). We tested this prediction in bacterial communities consisting of the strong competitor (PA wild type) and its inferior counterpart BC – a species assembly where the PA wild type typically drives BC to extinction (Figure 4a). We introduced strong intra-specific competition through the addition of a PA

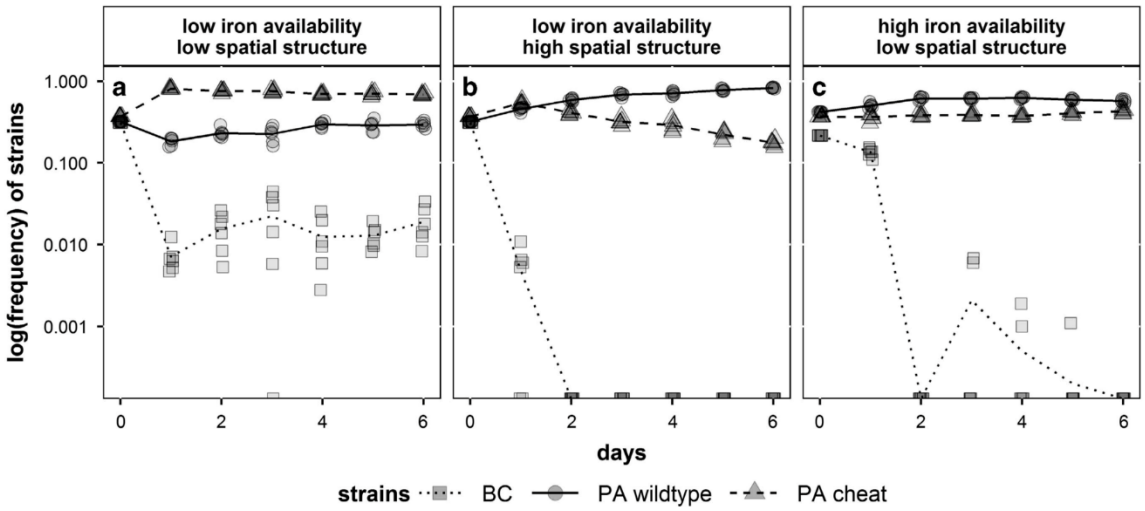


Figure 5 Evolutionary dynamics in three-strain communities consisting of BC (squares, dotted line), PA wild type (circles, solid line) and PA cheat (triangles, dashed line). BC was maintained at low frequency when iron availability and spatial structure were both low (**a**). In this scenario PA cheat could significantly outcompete the PA wild type and thereby buffer the competition between PA and BC. In contrast, species co-existence was neither possible when spatial structure was high (**b**) nor when iron availability was increased (**c**). In these scenarios, the PA cheat could not dominate the PA wild type, which led to the extinction of BC within 6 days. Symbols depict individual data points (from 6 replicates), whereas lines represent mean strain frequencies.

cheat strain, which exploited the publically shared iron-scavenging pyoverdine secreted by the PA wild type. We demonstrate that this form of intra-specific competition indeed favors species co-existence under well-mixed culturing conditions, where the PA cheat efficiently exploits pyoverdine, thereby reducing both the growth of the PA wild type and the associated suppression of BC. The equalizing fitness effect exerted by the PA cheat was, however, reduced with increased spatial structuring of the environment. This is because structuring prevents successful pyoverdine exploitation, such that the PA wild type remained dominant in the population, driving BC rapidly to extinction. Since the sharing and exploitation of secreted compounds are common in the natural microbial world (West *et al.*, 2007; Barrett *et al.*, 2011; Cordero *et al.*, 2012; Raymond *et al.*, 2012; Andersen *et al.*, 2015), we suggest that this form of intra-specific competition could take on an important role in maintaining biodiversity in habitats with relatively low spatial structure, such as marine ecosystems (Sogin *et al.*, 2006; Rusch *et al.*, 2007; Fuhrman *et al.*, 2008).

Our finding that cheating reverses the relationship between spatial structure and biodiversity contradicts a common paradigm in ecology (Tilman, 1994; Kerr *et al.*, 2002; Reichenbach *et al.*, 2007; Hibbing *et al.*, 2010; Celiker and Gore, 2012; Vos *et al.*, 2013; Coyte *et al.*, 2015). With regard to bacteria, our results particularly differ from those reported for toxin-mediated interference competition, where co-existence between toxin producers, susceptible and resistant strains has been shown to explicitly rely on spatial structure (Kerr *et al.*, 2002; Kirkup and Riley, 2004; Narisawa *et al.*, 2008; Biernaskie *et al.*, 2013; Pérez-Gutiérrez *et al.*, 2013; Abrudan *et al.*, 2015). The reason for this difference resides in the different types of social interactions involved. In the case of strong interference competition via toxins, spatial structure has a stabilizing fitness effect. It physically separates competing species, and thereby indirectly increases intra-specific competition. In contrast, public goods cheating has both a stabilizing (that is, increased intra-specific competition) and an equalizing (fitness of competing strains converge the more cheats there are) component. These two components are maximized with low spatial structure where public goods exploitation by cheats is most efficient (Figure 2c; Kümmerli *et al.*, 2009; Yang *et al.*, 2010; Mitri *et al.*, 2011). It is conceivable that the importance of the two social interaction types varies across habitat type: toxin-mediated interactions might be more relevant for biodiversity in well-structured soil- and surface-attached communities, whereas public goods cheating might be an important driver of biodiversity in relatively unstructured aquatic planktonic communities.

There are two recent studies, which present different mechanisms that can also maintain microbial biodiversity in the absence of significant spatial structure (Kelsic *et al.*, 2015; Inglis *et al.*, 2016). Kelsic *et al.* (2015) extended the classic models of toxin production (Czaran *et al.*, 2002; Kerr *et al.*, 2002) to include the cooperative degradation of toxins outside the cell, which offers immunity to resistant and susceptible strains alike. Their experimental work on agar plates showed that biodiversity can be stabilized by cooperative toxin degradation with high spatial structure. Their theoretical models suggest that the same mechanism could also stabilize

biodiversity in the absence of spatial structure. Inglis *et al.* (2016) on the other hand, established the loner effect, where cooperators and cheats can co-exist in the presence of a loner strain, which has an independent lifestyle and does not partake in social interactions with the other community members. In their system, loners beat cheats, cheats beat cooperators and cooperators beat loners, leading to cyclical rock-paper-scissors dynamics and strain co-existence. In principle, BC could act as a loner in our community fostering co-existence between cooperators and cheats. However, this is unlikely to be the case because the cheat-cooperator dynamics were unaffected by the presence of BC (Figures 4e and f and Figures 5a and b), and there was no evidence for cyclical succession of strains as observed in Inglis *et al.* (2016). Rather BC stayed at a constant low frequency in the population. This suggests that the cheating effect described here is a novel mechanism promoting species co-existence under low spatial structure.

In our scenario, community stability clearly depended on the relative success of cheats. Our results show that if the PA cheat cannot sufficiently supress the PA wild type then the weak competitor BC goes extinct (Figure 5). On the other hand, if the PA cheat were too efficient in exploiting the PA wild type then community stability would also not be possible, because the PA cheat would be expected to first displace the PA wild type, to then be displaced by BC. Thus, for cheating to sustain biodiversity in the long-term, an intermediate level of cheat success is required. There are three mutually non-exclusive factors that may contribute to an intermediate cheat success. First, the relative success of public good cheats often declines when they become more common, which can maintain cooperators at relatively low frequency in the population (Ross-Gillespie *et al.*, 2007; Gore *et al.*, 2009; Zhou *et al.*, 2014). Second, even in relatively well-mixed habitats, such as the ocean, there is some level of spatial structuring, which might prevent cheats from completely outcompeting cooperators (Cordero *et al.*, 2012; Kümmerli *et al.*, 2014). Finally, experimental evolution studies have shown that cooperators can rapidly adapt to the presence of cheats, thereby preventing cheats from fixing in the population (Zhang *et al.*, 2009; Kümmerli *et al.*, 2015).

While our results highlight that intra-specific competition can counterbalance inter-specific competition and foster species co-existence, we found that BC was typically maintained at rather low frequencies (that is, below 5%) in the community (Figure 5a). This finding is reminiscent of species abundance patterns in natural communities, where a few abundant species often occur together with an enormous diversity of rare species, the so-called rare biosphere (Sogin *et al.*, 2006; Rusch *et al.*, 2007). Although hypothetical at this stage, it would be interesting to test whether the cheating mechanism described in this paper can explain the occurrence and maintenance of the rare biosphere in natural habitats.

In conclusion, our study establishes cheating as a mechanism that can maintain biodiversity in habitats with low spatial structure. For this mechanism to work, cheating must be prevalent in dominant species but rare in subordinate species of the community. Although it remains to be seen whether this assumption indeed holds for natural multi-species communities, it seems a realistic assumption, as populations of dominant species are necessarily larger, which should favor the evolution of *de novo* cheats more readily due to higher mutation supply rates. Taken together, we show that cheating, which is typically considered as a destructive force in biology, undermining group-beneficial traits, can take on an important constructive role in shaping species co-existence at the level of the community.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We thank Martin Ackermann and Andy Gardner for comments, Leo Eberl for comments and providing strains, and the Swiss National Science Foundation for funding (grant no. PP00P3-139164).

Author contributions

AL and RK designed the research. AL conducted the experiments. AL, RFI and RK analyzed the data. AL, RFI and RK wrote the manuscript.

References

Abrudan MI, Smakman F, Grimbergen AJ, Westhoff S, Miller EL, van Wezel GP *et al.* (2015). Socially mediated induction and suppression of antibiosis during bacterial coexistence. *Proc Natl Acad Sci USA* **112**: 11054–11059.

Andersen SB, Marvig RL, Molin S, Johansen HK, Griffin AS. (2015). Long-term social dynamics drive loss of function in pathogenic bacteria. *Proc Natl Acad Sci USA* **112**: 10756–10761.

Barabás G, Michalska-Smith MJ, Allesina S. (2016). The effect of intra- and interspecific competition on coexistence in multispecies communities. *Am Nat* **188**: E1–E12.

Barrett LG, Bell T, Dwyer G, Bergelson J. (2011). Cheating, trade-offs and the evolution of aggressiveness in a natural pathogen population. *Ecol Lett* **14**: 1149–1157.

Becker J, Eisenhauer N, Scheu S, Jousset A. (2012). Increasing antagonistic interactions cause bacterial communities to collapse at high diversity. *Ecol Lett* **15**: 468–474.

Benjamini Y, Hochberg Y. (1995). Controlling the false discovery rate: a pratical and powerful approach to multiple testing. *J Roy Stat Soc Ser A* **57**: 289–300.

Biernaskie JM, Gardner A, West SA. (2013). Multicoloured greenbeards, bacteriocin diversity and the rock-paper-scissors game. *J Evol Biol* **26**: 2081–2094.

Bragonzi A, Farulla I, Paroni M, Twomey KB, Pirone L, Lore NI *et al.* (2012). Modelling co-infection of the cystic fibrosis lung by *Pseudomonas aeruginosa* and *Burkholderia cenocepacia* reveals influences on biofilm formation and host response. *PLoS One* **7**: e52330.

Brockhurst MA, Hochberg ME, Bell T, Buckling A. (2006). Character displacement promotes cooperation in bacterial biofilms. *Curr Biol* **16**: 2030–2034.

Celiker H, Gore J. (2012). Competition between species can stabilize public-goods cooperation within a species. *Mol Syst Biol* **8**: 621.

Chesson P. (2000). Mechanisms of maintenance of species diversity. *Annu Rev Ecol Evol Syst* **31**: 343–366.

Cordero OX, Ventouras L-A, DeLong EF, Polz MF. (2012). Public good dynamics drive evolution of iron acquisition strategies in natural bacterioplankton populations. *Proc Natl Acad Sci USA* **109**: 20059–20064.

Costello A, Reen FJ, O'Gara F, Callaghan M, McClean S. (2014). Inhibition of co-colonizing cystic fibrosis-associated pathogens by *Pseudomonas aeruginosa* and *Burkholderia multivorans*. *Microbiology* **160**: 1474–1487.

Coyte KZ, Schluter J, Foster KR. (2015). The ecology of the microbiome: Networks, competition, and stability. *Science* **350**: 663–666.

Curtis TP, Sloan WT, Scannell JW. (2002). Estimating prokaryotic diversity and its limits. *Proc Natl Acad Sci USA* **99**: 10494–10499.

Czaran T, Hoekstra RF, Pagie L. (2002). Chemical warfare between microbes promotes biodiversity. *Proc Natl Acad Sci USA* **99**: 786–790.

Dumas Z, Kümmerli R. (2012). Cost of cooperation rules selection for cheats in bacterial metapopulations. *J Evol Biol* **25**: 473–484.

Dumas Z, Ross-Gillespie A, Kümmerli R. (2013). Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. *Proc Biol Sci* **280**: 20131055.

Foster KR, Bell T. (2012). Competition, not cooperation, dominates interactions among culturable microbial species. *Curr Biol* **22**: 1845–1850.

Fuhrman JA, Steele JA, Hewson I, Schwalbach MS, Brown MV, Green JL *et al.* (2008). A latitudinal diversity gradient in planktonic marine bacteria. *Proc Natl Acad Sci USA* **105**: 7774–7778.

Gore J, Youk H, van Oudenaarden A. (2009). Snowdrift game dynamics and facultative cheating in yeast. *Nature* **456**: 253–256.

Gotschlich A, Huber B, Geisenberger O, Togl A, Steidle A, Riedel K *et al.* (2001). Synthesis of multiple N-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. *Syst Appl Microbiol* **24**: 1–14.

Griffin A, West SA, Buckling A. (2004). Cooperation and competition in pathogenic bacteria. *Nature* **430**: 1024–1027.

Hardin G. (1960). The competitive exclusion principle. *Science* **131**: 1292–1297.

Harrison F, Paul J, Massey RC, Buckling A. (2008). Interspecific competition and siderophore-mediated cooperation in *Pseudomonas aeruginosa*. *ISME J* **2**: 49–55.

Hersman LE, Huang A, Maurice PA, Forsythe JE. (2000). Siderophore production and iron reduction by *Pseudomonas mendocina* in response to iron deprivation. *Geomicrobiol J* **17**: 261–273.

Hibbing ME, Fuqua C, Parsek MR, Peterson SB. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* **8**: 15–22.

Holt RD, Grover J, Tilman D. (1994). Simple rules for interspecific dominance in systems with exploitative and apparent competition. *Am Nat* **144**: 741–771.

Inglis RF, Biernaskie JM, Gardner A, Kümmerli R. (2016). Presence of a loner strain maintains cooperation and diversity in well-mixed bacterial communities. *Proc Biol Sci* **283**: 20152682.

Kelsic ED, Zhao J, Vetsigian K, Kishony R. (2015). Counteraction of antibiotic production and degradation stabilizes microbial communities. *Nature* **521**: 516–519.

Kerr B, Riley MA, Feldman MW, Bohannan BJM. (2002). Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. *Nature* **418**: 171–174.

Kim HJ, Boedicker JQ, Choi JW, Ismagilov RF. (2008). Defined spatial structure stabilizes a synthetic multispecies bacterial community. *Proc Natl Acad Sci USA* **105**: 18188–18193.

Kirkup BC, Riley MA. (2004). Antibiotic-mediated antagonism leads to a bacterial game of rock–paper–scissors *in vivo*. *Nature* **428**: 412–414.

Kümmerli R, Griffin AS, West SA, Buckling A, Harrison F. (2009). Viscous medium promotes cooperation in the pathogenic bacterium *Pseudomonas aeruginosa*. *Proc Biol Sci* **276**: 3531–3538.

Kümmerli R, Schiessl KT, Waldvogel T, McNeill K, Ackermann M. (2014). Habitat structure and the evolution of diffusible siderophores in bacteria. *Ecol Lett* **17**: 1536–1544.

Kümmerli R, Santorelli L, Granato E, Dumas Z, Dobay A, Griffin AS *et al.* (2015). Co-evolutionary dynamics between public good producers and cheats in the bacterium *Pseudomonas aeruginosa*. *J Evol Biol* **28**: 2264–2274.

Lambiase A, Raia V, Del Pezzo M, Sepe A, Carnovale V, Rossano F. (2006). Microbiology of airway disease in a cohort of patients with cystic fibrosis. *BMC Infect Dis* **6**: 4.

Lankau RA. (2009). Genetic variation promotes long-term coexistence of *Brassica nigra* and its competitors. *Am Nat* **174**: E40–E53.

Miethke M, Marahiel MA. (2007). Siderophore-based iron acquisition and pathogen control. *Microbiol Mol Biol Rev* **71**: 413–451.

Mitri S, Xavier JB, Foster KR. (2011). Social evolution in multispecies biofilms. *Proc Natl Acad Sci USA* **108**: 10839–10846.

Narisawa N, Haruta S, Arai H, Ishii M, Igarashi Y. (2008). Coexistence of antibiotic-producing and antibiotic-sensitive bacteria in biofilms is mediated by resistant bacteria. *Appl Environ Microbiol* **74**: 3887–3894.

Pérez-Gutiérrez RA, López-Ramírez V, Islas Á, Alcaraz LD, Hernández-González I, Olivera BCL *et al.* (2013). Antagonism influences assembly of a *Bacillus* guild in a local community and is depicted as a food-chain network. *ISME J* **7**: 487–497.

R Development Core Team. (2015). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing: Vienna, Austria.

Rainey PB, Travisano M. (1998). Adaptive radiation in a heterogeneous environment. *Nature* **394**: 69–72.

Rainey PB, Rainey K. (2003). Evolution of cooperation and conflict in experimental bacterial poplations. *Nature* **425**: 72–74.

Raymond B, West SA, Griffin AS, Bonsall MB. (2012). The dynamics of cooperative bacterial virulence in the field. *Science* **337**: 85–88.

Reichenbach T, Mobilia M, Frey E. (2007). Mobility promotes and jeopardizes biodiversity in rock-paper-scissors games. *Nature* **448**: 1046–1049.

Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng J-F *et al.* (2013). Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**: 431–437.

Rosenzweig RF, Sharp RR, Treves DS, Adams J. (1994). Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli*. *Genetics* **137**: 903–917.

Ross-Gillespie A, Gardner A, West SA, Griffin AS. (2007). Frequency dependence and cooperation: theory and a test with bacteria. *Am Nat* **170**: 331–342.

Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S *et al.* (2007). The Sorcerer II Global Ocean Sampling expedition: northwest Atlantic through eastern tropical Pacific. *PLoS Biol* **5**: e77.

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR *et al.* (2006). Microbial diversity in the deep sea and the underexplored ''rare biosphere''. *Proc Natl Acad Sci USA* **103**: 12115–12120.

Sunagawa S, Coelho LP, Chaffron S, Kultima JR, Labadie K, Salazar G *et al.* (2015). Structure and function of the global ocean microbiome. *Science* **348**: 1261359.

The Human Microbiome Project. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* **486**: 207–214.

Tilman D. (1994). Competition and biodiversity in spatially structured habitats. *Ecology* **75**: 2–16.

Tyrrell J, Callaghan M. (2015). Iron acquisition in the Cystic Fibrosis lung and potential for novel therapeutic strategies. *Microbiology* **162**: 191–205.

Tyrrell J, Whelan N, Wright C, Sa-Correia I, McClean S, Thomas M *et al.* (2015). Investigation of the multifaceted iron acquisition strategies of *Burkholderia cenocepacia*. *Biometals* **28**: 367–380.

Vos M, Wolf AB, Jennings SJ, Kowalchuk GA. (2013). Micro-scale determinants of bacterial diversity in soil. *FEMS Microbiol Rev* **37**: 936–954.

Weaver BW, Kolter R. (2004). *Burkholderia* spp. alter *Pseudomonas aeruginosa* physiology through iron sequestration. *J Bacteriol* **186**: 2376–2384.

West SA, Diggle SP, Buckling A, Gardner A, Griffin AS. (2007). The social lives of microbes. *Annu Rev Ecol, Evol Syst* **38**: 53–77.

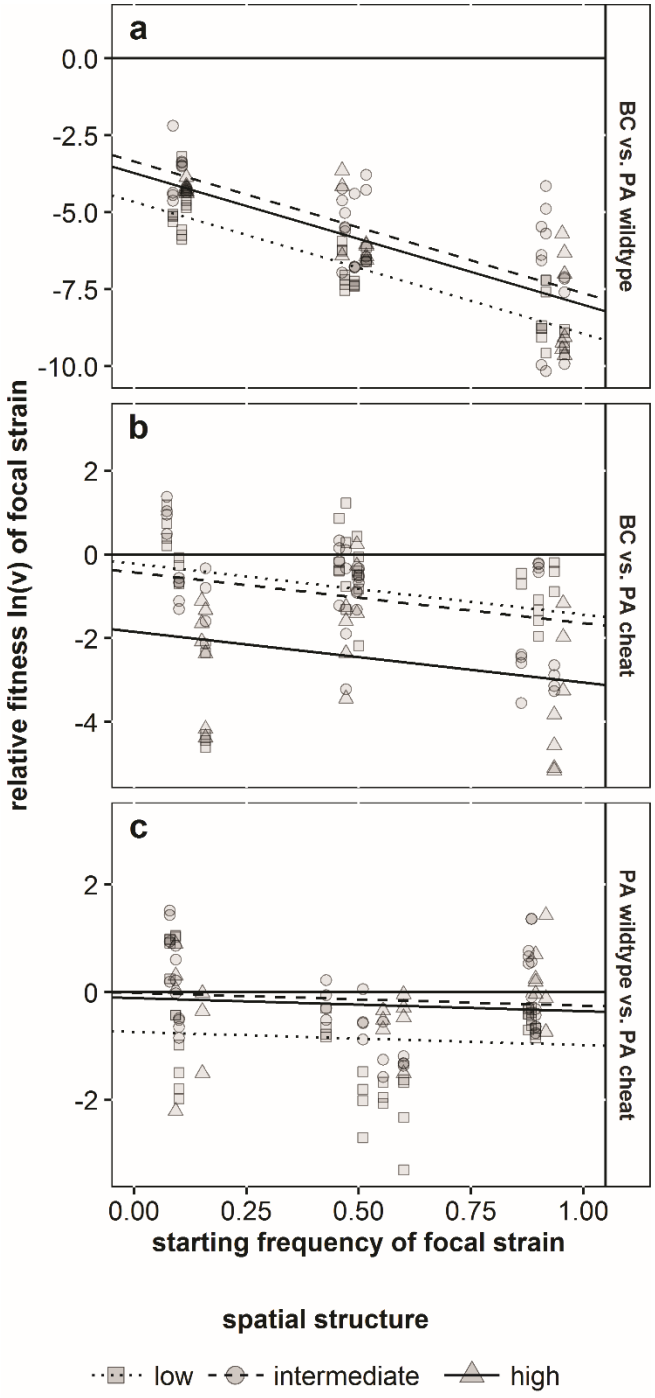
Yang J, Evans BA, Rozen DE. (2010). Signal diffusion and the mitigation of social exploitation in pneumococcal competence signalling. *Proc Biol Sci* **277**: 2991–2999.

Zhang Q-G, Buckling A, Ellis RJ, Godfray HCJ. (2009). Coevolution between cooperators and cheats in a microbial system. *Evolution* **63**: 2248–2256.

Zhou L, Slamti L, Nielsen-LeRoux C, Lereclus D, Raymond B. (2014). The social biology of quorum sensing in a naturalistic host pathogen system. *Curr Biol* **24**: 2417–2422.

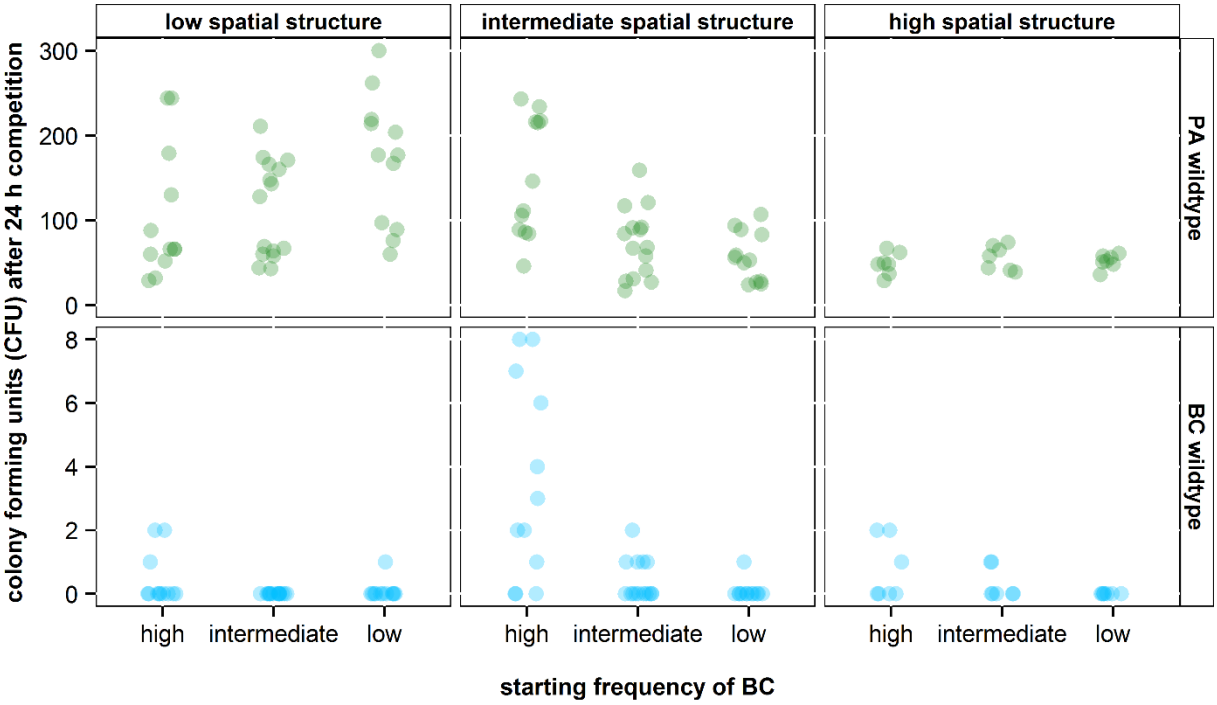
Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

3.1. Supporting Information

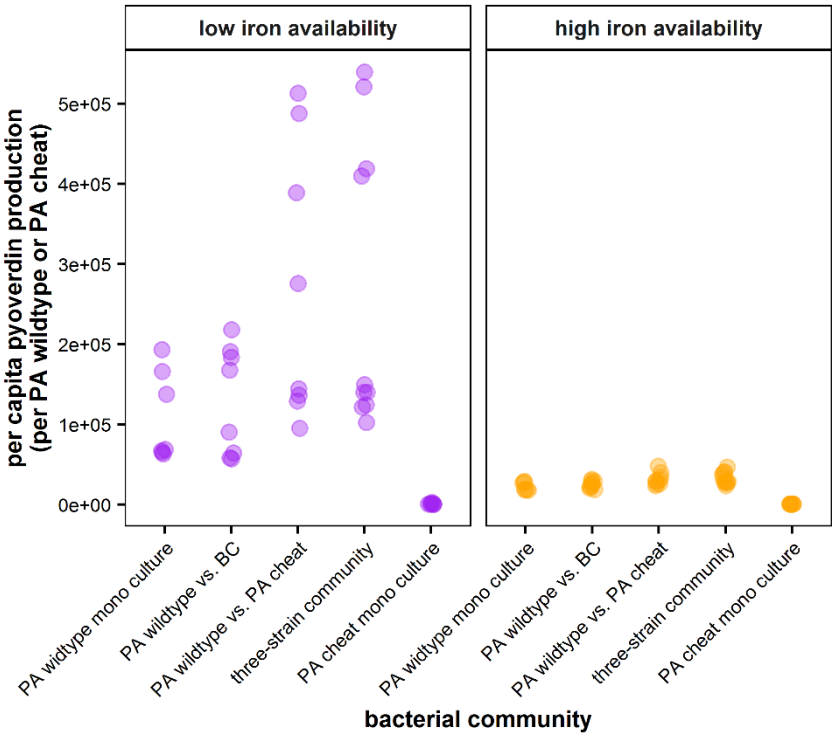


***Supporting Figure 1**: Relative fitness ln(v) in pairwise competitions between BC, PA wildtype and PA cheat in iron-limited CAA medium across different starting frequencies. We varied the starting frequencies to examine the robustness of competitive outcomes across a range of mixing ratios. We found that BC is outcompeted by the PA wildtype over the range of all tested starting frequencies. Our data suggest that the relative fitness of BC is negative frequency dependent (LM: slope = -4.28, $F_{1,95}$ = 168.16, $p < 0.0001$). This pattern is explained by the fact that BC almost always went extinct, such that its relative fitness is maximized at high starting frequencies (a). We further demonstrate that the PA cheat is a weaker competitor than the PA wildtype against BC. Though BC is significantly outcompeted by the PA cheat at all starting frequencies, BC performs significantly better when it is rare (test for negative-frequency dependence, LM: slope = -1.21, $F_{1,92}$ = 9.36, $p = 0.0075$) (b). The relative fitness of the PA wildtype when competing against the PA cheat does not significantly correlate with its starting frequency (LM: slope = -0.242, $F_{1,95}$ = 0.59, $p = 0.443$) (c). Thus, we do not expect the starting frequency of the PA cheat to have an effect on the maintenance of biodiversity (at least not over the range tested here). The relative fitness ln(v) of the*

focal strain (the first strain in the panel headers) is shown for all three spatial structures (square = low, circle = intermediate, triangle = high). Relative fitness values of ln(v) < 0, ln(v) > 0, or ln(v) = 0, indicate whether the focal strain respectively lost, won, or performed equally well in competition with its opponent. Grey symbols represent individual data points, whereas black symbols indicate the mean ± 95% CI. Trend lines represent the estimated associations between ln(v) and the starting frequency, obtained from our linear models.



***Supporting Figure 2**: Counts of colony forming units (CFU) were drastically lower for BC (blue) than for PA (green) after a 24-hours competition period. The competition assays were performed in iron-limited CAA medium with different spatial structures and starting frequencies. The dots represent replicate competition assays. The number of replicates was at least 12 for competitions with low and intermediate spatial structure, and at least 7 for competition with high spatial structure.*



***Supporting Figure 3**: PA wildtype produces lower levels of pyoverdine in CAA medium where iron is readily available compared to CAA medium with low iron availability. Shown are the levels of pyoverdine production per OD units. For mixed cultures, the OD units were corrected for the frequency of the PA wildtype after the competition. The pyoverdine level was measured as fluorescent units (FU) (excitation wavelength = 400 nm, emission wavelength = 460 nm) in a Tecan Infinite M-200 plate reader (Tecan Group Ltd., Switzerland). Each dot represents an independent data point.*

***Supporting Table 1**: Colony forming units (CFU), frequency and detection limit of BC after 24 h competition with PA wildtype in medium with different spatial structures and low iron availability*

competition	spatial structure	starting frequency (mean ± SE)	**minimal [a] detection limit of BC**	minimal frequency of BC	maximal frequency of BC	mean frequency of BC	minimal total CFUs	maximal total CFUs	mean total CFUs	minimal CFUs of BC	maximal CFUs of BC	mean CFUs of BC	no. of replicates with CFU of BC = 0
PA wt vs. BC	low	0.9273 ± 0.0067	**0.0077**	0.0000	0.0081	0.0018	29	246	105.083	0	2	0.4167	9 of 12
PA wt vs. BC	low	0.4865 ± 0.0056	**0.0033**	0.0000	0.0000	0.0000	43	211	113.733	0	0	0.0000	15 of 15
PA wt vs. BC	low	0.1030 ± 0.0037	**0.0033**	0.0000	0.0049	0.0004	60	300	170.250	0	1	0.0833	11 of 12
PA wt vs. BC	Inter-mediate	0.9273 ± 0.0067	**0.0043**	0.0000	0.1481	0.0315	54	247	152.833	0	8	3.4167	3 of 12
PA wt vs. BC	Inter-mediate	0.4865 ± 0.0056	**0.0083**	0.0000	0.0238	0.0047	17	161	73.067	0	2	0.4000	10 of 15
PA wt vs. BC	Inter-mediate	0.1030 ± 0.0037	**0.0093**	0.0000	0.0105	0.0009	24	107	58.000	0	1	0.0833	11 of 12
PA wt vs. BC	high	0.9561 ± 0.0011	**0.0149**	0.0000	0.0645	0.0178	31	67	49.429	0	2	0.7143	4 of 7
PA wt vs. BC	high	0.4937 ± 0.0108	**0.0133**	0.0000	0.0222	0.0051	39	75	56.143	0	1	0.2857	5 of 7
PA wt vs. BC	high	0.1165 ± 0.0001	**0.0164**	0.0000	0.0000	0.0000	36	61	51.714	0	0	0.0000	7 of 7

a: For calculations a CFU of BC = 0 was substituted with 1. Then the frequency of BC of the total population was calculated. The minimal frequency value of all replicates equals the minimal detection limit.

***Supporting Table 2**: Colony forming units (CFU), frequency and detection limit of BC at day 6 of experimental evolutions*

competition	spatial structure	iron availability	minimal total CFUs	maximal total CFUs	mean total CFUs	**minimal [a] detection limit of BC**	minimal frequency of BC	maximal frequency of BC	mean frequency of BC	minimal CFUs of BC	maximal CFUs of BC	no. of replicates with CFU of BC = 0
PA wt vs. BC	low	low	244	353	295.3	**0.28%**	0.00%	0.34%	0.08%	0	1	3 of 4
PA wt vs. BC	high	low	245	379	306.3	**0.26%**	0.00%	0.00%	0.00%	0	0	4 of 4
three-strain competition	low	low	242	370	298.3	-	0.83%	3.36%	1.89%	2	10	0 of 6
three-strain competition	high	low	267	391	334.5	**0.26%**	0.00%	0.00%	0.00%	0	0	6 of 6
three-strain competition	low	high	220	800	492.8	**0.13%**	0.00%	0.00%	0.00%	0	0	6 of 6

a: For calculations a CFU of BC = 0 was substituted with 1. Then the frequency of BC of the total population was calculated. The minimal frequency value of all replicates equals the minimal detection limit.

4. Second Project: The bacterium Pseudomonas aeruginosa senses and gradually responds to inter-specific competition for iron

This research is in the resubmission stage for the journal “Evolution”.

Authors: **Anne Leinweber[1], Michael Weigert[1, 2], Rolf Kümmerli[1]**

[1] Department of Plant and Microbial Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

[2] Microbiology, Department of Biology I, Ludwig Maximilians University Munich, Grosshaderner Strasse 2-4, 82152 Martinsried, Germany.

Abstract

Phenotypic plasticity in response to competition is a well-described phenomenon in higher organisms. Here, we show that also bacteria have the ability to sense the presence of competitors and mount fine-tuned responses to match prevailing levels of competition. In our experiments, we studied inter-specific competition for iron between the bacterium *Pseudomonas aeruginosa* (PA) and its competitor *Burkholderia cenocepacia* (BC). We focused on the ability of PA to phenotypically adjust the production of pyoverdine, an iron-scavenging siderophore. We found that PA up-regulates pyoverdine production during the early phase of competition against BC. This plastic upregulation was fine-tuned in response to the level of competition, and conferred a fitness benefit in the form of an earlier initiation of growth. Our fitness analysis further points towards a trade-off: mounting a costly early response might confer a relative fitness advantage over competitors, but curbs absolute fitness at later time points. Taken together, our results demonstrate that phenotypic plasticity in siderophore production plays an important role in inter-specific competition for iron. Upregulating siderophore production seems to be a powerful strategy to lock away iron from competing species, and to reserve this nutrient for strain members possessing the compatible receptor for uptake.

Key words: inter-specific competition, phenotypic plasticity, microbe-microbe interactions, siderophores, fitness trade-off

Introduction

Under natural conditions, bacteria live in diverse communities, where competition for space and nutrients is intense (Whitman et al. 1998; Curtis et al. 2002; Becker et al. 2012; Foster and Bell 2012; Rinke et al. 2013). Bacterial traits, such as the secretion of toxins, the formation of protective biofilms, and the efficient scavenging of nutrients are evidently interpreted as direct evolutionary adaptations to inter-specific competition (Weaver and Kolter 2004; Harrison et al. 2008; Bakkal et al. 2010; Tashiro et al. 2013; Oliveira et al. 2015). Less well understood, however, is to what extent bacteria can sense different levels of competition and adjust their phenotype accordingly. Because the expression of a 'competitive phenotype' is costly, it would make sense to solely express it when required, and to adjust its expression to match the prevailing level of competition. Phenotypic plasticity in response to competition is well documented for higher organisms (Agrawal 2001; Boege 2010; Leggett et al. 2014; Cortesi et al. 2015), and has recently also been put forward for bacteria (Cornforth and Foster 2013). Phenotypic plasticity, defined as the ability of a single genotype to express different phenotypes (West-Eberhard 1989), allows fast responses to environmental changes, which is thought to be adaptive, especially in fluctuating environments (Fordyce 2006).

Here, we use a laboratory model system to examine whether the bacterium *Pseudomonas aeruginosa* (PA) can sense and plastically respond to inter-specific competition for iron, when growing in co-culture with *Burkholderia cenocepacia* (BC). Iron is an essential nutrient and growth-limiting factor in most natural environments because ferric iron is insoluble at neutral pH or host-bound in the context of infections (Hersman et al. 2000; Miethke and Marahiel 2007). To overcome iron limitation, bacteria secrete siderophores, small secondary metabolites, which make ferric iron bio-available for all individuals with a specific receptor for uptake (Ratledge and Dover 2000; Miethke and Marahiel 2007; Tyrrell and Callaghan 2015). Previous research showed that siderophores play a crucial role for both intra- and inter-specific competition (Griffin et al. 2004; Weaver and Kolter 2004; West et al. 2006; Ross-Gillespie et al. 2007; Harrison et al. 2008; Kümmerli et al. 2009a; Traxler et al. 2013; Leinweber et al. 2017). In intra-specific competition, the secreted siderophores can be shared as public goods between cells that possess a compatible receptor for uptake. Sharing can select for cheating by mutants that no longer produce siderophores themselves, yet still capitalize on the siderophores produced by others (Griffin et al. 2004; West et al. 2006; Ross-Gillespie et al. 2007, 2015; Kümmerli et al. 2009a; Scholz and Greenberg 2015). For inter-specific competition, meanwhile, the secretion of large quantities of siderophores could represent an efficient way to outpace the competing species in the race for iron, because inter-specific competitors typically have different, incompatible siderophore systems (Weaver and Kolter 2004; Harrison et al. 2008; Schiessl et al. 2016; Leinweber et al. 2017; Niehus et al. 2017). An alternative competitive strategy would be to produce a siderophore with

particularly high iron affinity, which would allow to directly steal iron that is bound to the competing species' siderophores (Joshi et al. 2006).

To assess the role of siderophores for inter-specific competition, we carried out experiments in mixed-species communities, with PA as our focal strain and BC as its competitor. We addressed three main questions. First, we asked whether PA adjusts the production of its primary siderophore, pyoverdine, in response to inter-specific iron competition. To tackle this question, we grew PA in mono- and mixed culture with BC in three different environments varying in their level of iron limitation, and quantified the variation in PA's pyoverdine production profiles. Second, we asked whether phenotypic plasticity in response to competition involves a binary switch from one phenotype to another (i.e. on/off competition stage) or whether responses can be more gradually adjusted to match the prevailing level of competition. To answer this question, we exposed PA to various levels of BC competition by modifying the mixing ratio of the two species. Since BC is a relatively weak competitor, which produces a less efficient siderophore (ornibactin) than PA (Leinweber et al. 2017), we hypothesized that PA should primarily adjust its pyoverdine phenotype when competitive pressure from BC is high. Finally, we were interested in exploring whether phenotype adjustments in pyoverdine production have fitness consequences for PA. This is a crucial point because if competition sensing and the resulting change in phenotype were adaptive then adjustment of siderophore phenotypes should entail fitness benefits.

Methods

Bacterial strains, media, and growth conditions

For all our experiments, we used the standard laboratory *P. aeruginosa* strain PAO1 (ATCC 15692) and *B. cenocepacia* strain H111 (LMG 23991), an isolate from a cystic fibrosis patient (Gotschlich et al. 2001). To be able to distinguish PA from BC in mixed cultures, we used a PA variant that constitutively expressed *mcherry* (chromosomal insertion *att*Tn7::Ptac-*mcherry*) for all experiments. H111 did not carry a fluorescent marker. For some experiments, we further used the reporter strain PAO1*pvdA-gfp*, where *gfp* expression is under the control of the *pvdA*-promoter (chromosomal insertion *att*B::pvdA) (Kaneko et al. 2007) and which also featured a constitutively expressed mCherry marker (chromosomal insertion *att*Tn7::Ptac-*mcherry*). Since PvdA catalyses a key step in the production of pyoverdin (Visca et al. 1994; Leoni et al. 1996), the expression level of *pvdA* serves as a proxy for the overall level of pyoverdin production.

Overnight cultures were grown in lysogeny broth (LB). All experiments were conducted in casamino acids (CAA) medium (per 1 liter: 5 g casamino acids; 1.18 g $K_2HPO_4 \cdot 3H_2O$; 0.25 g $MgSO_4 \cdot 7H_2O$)

supplemented with 20 mM $NaHCO_3$ and 25 mM HEPES buffer. We induced strong iron limitation by adding 100 µg/ml of the natural iron chelator human apo-transferrin. Intermediate iron levels were achieved by adding 0.5µM $FeCl_3$ to CAA medium with 100 µg/ml human apo-Transferrin. Previous results have shown that PA halves pyoverdine production under these conditions (Kümmerli et al. 2009b). To create iron-replete medium, we omitted transferrin from the above recipe and added 20µM $FeCl_3$ instead. All chemicals were purchased from Sigma-Aldrich, Switzerland.

Measuring pyoverdine production profiles in response to competition

We designed a multi-factorial experiment to test whether PA can sense inter-specific competition for iron and adjust its pyoverdine production accordingly. The first factor compared the pyoverdine production profiles of PA when grown in mono- versus mixed culture with BC. The second factor focused on the level of competition between the two species, and addressed the question whether PA gradually adjusts its pyoverdine production phenotype when exposed to increasing frequencies of BC (i.e. higher levels of competition). The third factor examined the effect of iron availability on pyoverdine production profiles. The prediction here is that phenotypic plasticity in response to iron competition should mainly manifest at low iron availability, when competition for this nutrient is most intense.

Prior to the experiment, PA and BC were grown overnight in LB medium, harvested by centrifugation (7500 rpm, 2 min), and washed twice with 0.8% NaCl solution. Cultures were then adjusted to OD_{600} = 1 (optical density at 600 nm). Subsequently, we mixed PA and BC in different volumetric ratios (0.5% to 99.5%; 1% to 99%; 5% to 95%; 10% to 90%; 50% to 50%, respectively) for the inter-specific competition treatments. To obtain the same initial cell densities of PA in monocultures, we mixed PA with 0.8% NaCl, instead of BC, using the same volumetric ratios as indicated above. These bacterial cultures were then inoculated into CAA medium (featuring the three iron concentrations described above) in sterile 2 ml reaction tubes (Sarstedt) to an initial OD_{600} of $1x10^{-2}$. We then distributed the inoculated medium to 96-well plates (200 µl per well) in 7 to 8 replicates per treatment, and incubated the cultures in a Tecan Infinite M-200 plate reader (Tecan Group Ltd., Switzerland) at 37°C. To quantify pyoverdine production profiles, we measured the auto-fluorescence of pyoverdin (excitation = 400 nm, emission = 460 nm) every 15 min for 48 h. Prior to each reading event, plates were shaken for 30 seconds (3.5 mm orbital displacement).

To analyze the pyoverdine production profiles, we fitted non-parametric spline models to the fluorescence data, using the grofit R package (Kahm et al. 2010). We used spline fitting because pyoverdine trajectories followed non-logistic patterns and varied greatly between the different

treatments (Figure S1). We took the integral of the spline fits (i.e. area under the curve) as a proxy for the cumulative pyoverdine availability over time, and for comparisons of the global pyoverdine production profiles between the different treatments (i.e. mono vs. mixed culture, level of competition, iron concentration). To obtain differences in the temporal dynamics of pyoverdine production between mono and mixed cultures, we calculated the mean difference of pyoverdine fluorescence across replicates for each time point and treatment as: *mean pyoverdine fluorescence of mixed culture – mean fluorescence of the respective monoculture*. To estimate standard errors of this concatenated variable, we applied the jackknife function from the bootstrap R package (Tibshirani and Leisch 2015).

Measuring growth trajectories in mono- and mixed cultures

To follow PA growth trajectories in mixed and monocultures, we used the same experimental setup described above. Since optical density (the standard measure for growth) cannot be used to follow PA growth in mixed cultures with BC, we relied on the constitutively expressed mCherry marker to track PA growth. We measured the mCherry fluorescence (excitation = 582 nm, emission = 620 nm) of PA every 15 min for 48 h with the plate reader under the same culturing conditions as described above.

We used Gompertz models from the grofit package (Kahm et al. 2010) to estimate three growth parameters adequately describing the observed growth trajectories across treatments (Figure S2). These are: (a) the initial phase (IP), which describes the part of the lag-period, where growth rate equals zero (Prats et al. 2008); (b) the maximum growth rate μ, which is the maximal slope of the growth curve; and (c) the peak value representing the yield.

Single cell pvdA gene expression analysis

The above experiments will allow us to establish correlations between pyoverdine levels and growth trajectories. However, the actual causality between the two variables will remain concealed. For example, it is possible that: (i) a higher pyoverdine production level per cell leads to accelerated growth; or (ii) cells proliferate faster due to other reasons, which would result in higher pyoverdine availabilities at the population level without individual cells actually changing their pyoverdine production. To disentangle these two options, we directly quantified the expression of *pvdA* (a gene involved in pyoverdine synthesis) of PA cells in mono and mixed cultures with BC over time using fluorescence microscopy. To prepare microscopy samples, we first grew monocultures of the PA reporter strain (PAO1 *pvdA-gfp mcherry*) and BC in LB overnight and subsequently harvested cells by

centrifugation at 7500rpm, followed by two washing steps in 0.8% NaCl. The cultures were adjusted to OD_{600} = 1 and mixed in a ratio of 0.5% PA and 99.5% BC. For PA monocultures, BC was substituted with an equivalent volume of 0.8% NaCl. Iron limited CAA medium (supplemented with 100 µg/ml transferrin) was then inoculated with PA only or a mixture of PA and BC, and iron replete CAA medium (supplemented with 100 µM $FeCl_3$) was inoculated with PA only, in four 96-well plates (200µl per well) to a starting OD_{600} of $1x10^{-2}$. The plates were incubated at 37°C and 170 rpm in an orbital shaker. At each of the four time points (10, 12, 14 and 20 hours after inoculation), we processed one of the 96-well plates for microscopy. Specifically, we diluted the grown cultures in 0.8% NaCl to appropriate levels (Supplementary Table 1) and then added 1 µl of the culture onto a 1% agarose pad (in 0.8% NaCl) on a standard microscopy slide (agarose pad preparation followed the protocol described in (Weigert and Kümmerli 2017). For all the mCherry positive cells (expressed by the PA reporter strain only), we measured the *pvdA* associated GFP fluorescence. Imaging was performed with a widefield Leica DMI6000 microscope equipped with a plan APO PH3 objective (NA = 1.3), an automated stage and a Leica DFC 350 FX camera (resolution: 1392x1040 pixels) for image recording (16 bit color depth). We used a Leica L5 filter cube for GFP (emission: 480 ± 40 nm, excitation: 527 ± 30 nm, DM = 505) and a Leica TX2 filter cube for mCherry (emission: 560 ± 40 nm, excitation: 645 ± 75 nm, DM = 595) fluorescence. The exposure time for measuring GFP-fluorescence was 268 ms and for mCherry 182 ms with a (halogen) lamp intensity of 100%. We analyzed between 144 and 642 mCherry-positive PA cells per treatment and time point (see Supplementary Table 1 for detailed information on sample size). Single cell analysis followed the method described in (Weigert and Kümmerli 2017). For image segmentation we used the supervised object classification and segmentation tool ilastik (Sommer et al. 2011). Images were then further analyzed with Fiji, a free scientific image processing software package (Schindelin et al. 2012).

Statistical analysis

All statistical analyses and model fitting was carried out in R 3.1.2 (R Development Core Team 2015).

We used analysis of covariance (ANCOVA) to test for significant effects of the three following variables and their interactions. We included the degree of iron limitation and the absence/presence of the competitor BC as categorical variables, and the level of competition (Figure 1 and 3) or time (Figure 4) as covariates into our models. While we worked with complete models for the analysis of the overall pyoverdine production (Figure 1) and *pvdA* expression over time (Figure 4), we constructed separate models for each growth parameter and degree of iron limitation for the fitness analysis (Figure 3). We applied two-sample *t*-tests, to analyze whether pyoverdine production profiles differ between mono-

and mixed cultures at specific mixing ratios (Figure 1), or whether *pvdA* expression differs at specific time points (Figure 4). We used the Shapiro-Wilk-Test to check for normal distribution of the model residuals, prior to all analyses.

Results

Iron, the presence of competitors, and the level of competition affect pyoverdine production

Our multi-factorial analysis revealed high phenotypic plasticity in pyoverdine production by PA in response to iron concentration, the absence/presence of a competitor, and the level of competition between PA and BC (Figure 1). Consistent with previous studies (Kümmerli et al. 2009b; Dumas et al. 2013), our data show that PA gradually increases pyoverdine production when iron becomes more restricted (Linear model (LM): $F_{2, 220} = 5702.32$, $p < 0.0001$; Figure 1 + S1). Moreover, we found that the presence of the competitor BC significantly reduced global pyoverdine levels in mixed compared to PA monocultures, with the effect being significantly more pronounced with stronger competition (LM: significant interaction between the initial PA ratio and the competition treatment (mixed vs. monoculture), $F_{1,220}=22.408$, $p<0.0001$; Figure 1 + S1).

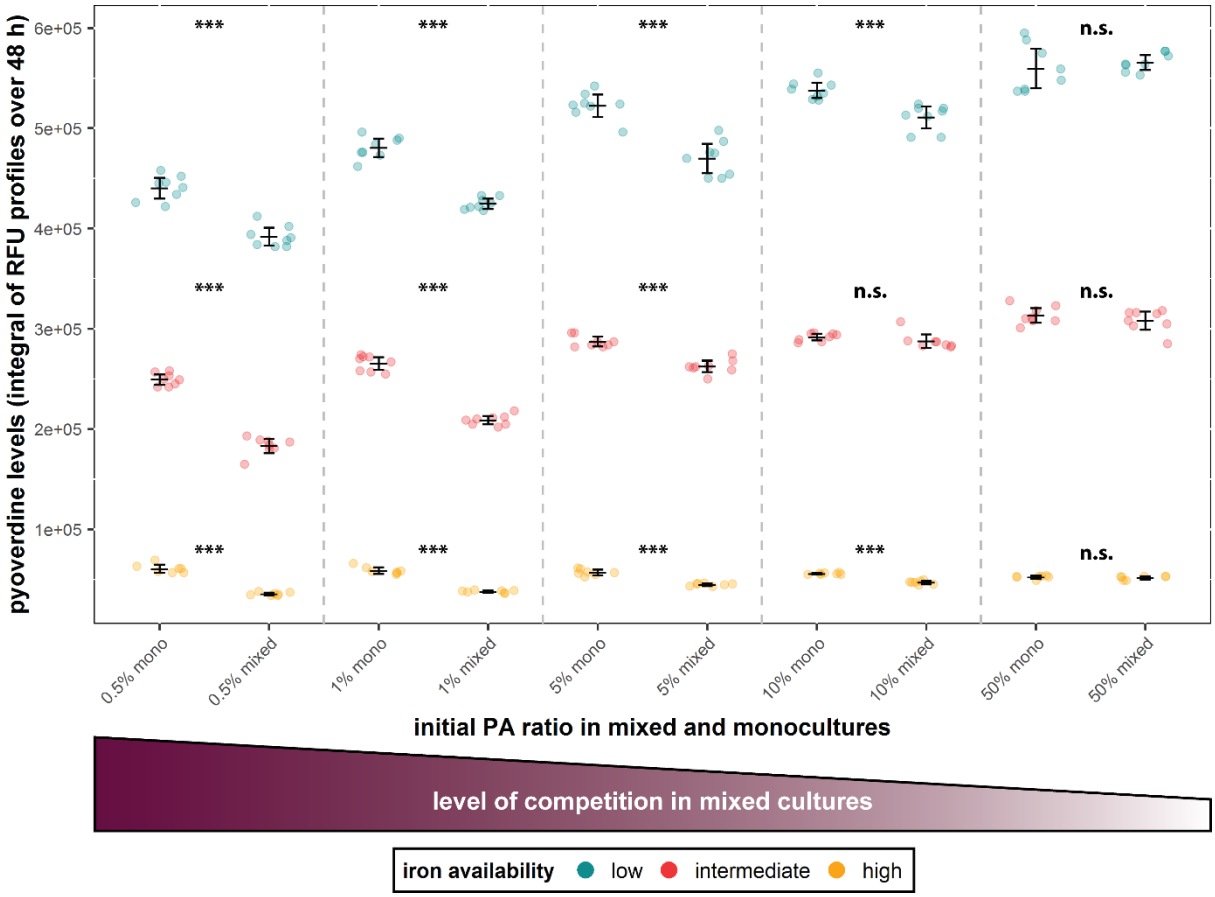


Figure 1**: The global pyoverdine level in P. aeruginosa (PA) cultures is influenced by inter-specific competition with B. cenocepacia (BC) and iron availability in the medium. High levels of competition with BC reduce overall pyoverdine levels compared to the respective PA monocultures, whereas pyoverdine levels converge in mixed compared to monocultures when competition levels decline (illustrated by the purple bar). Across all levels of competition, pyoverdine levels increase when iron availability decreases. In mixed cultures, we manipulated the level of competition exerted by BC on PA by varying the starting ratio of BC (50-99.5%), and inoculated respective PA monocultures with the same initial PA cell densities. Bacterial cultures were grown in CAA media under low (blue), intermediate (red) and high (orange) iron availability. Circles depict individual data points (7 to 8 replicates per treatment) and values represent the integral of the global pyoverdine production profile (area under the curve across 48 hours). Black symbols depict means ± 95% confidence intervals. Asterisks and n.s. indicate significant (P<0.0001) and non-significant differences between PA in mono- versus mixed culture, respectively.*

Temporal variation in pyoverdine profiles in response to competition

While the above analysis focused on overall pyoverdine availability across 48 hours, we here focus in more detail on the temporal variations in pyoverdine levels within and across treatments. As before, we found that iron availability, the absence/presence of a competitor, and the level of inter-specific competition all affected temporal pyoverdine availabilities (Figure 2). Most strikingly, we observed higher pyoverdine levels in mixed compared to monocultures during the early competition phase, particularly under iron limitation and strong competition (positive peaks in Figure 2a). The amplitude of the pyoverdine peaks gradually declined with decreasing levels of competition. While small pyoverdine peaks were also detectable at intermediate iron availability (Figure 2b), they completely disappeared under iron-replete conditions (Figure 2c). Overall, the positive peaks suggest that PA

upregulates pyoverdine production during the early competition phase against BC, and the upregulation is fine-tuned in response to the level of competition and iron availability.

At later stages of the competition, we also observed pronounced temporal variation in pyoverdine levels between treatments. But this time the monocultures show higher pyoverdine levels (negative peaks in Figure 2). As for the early phase of competition, we observed that the amplitude of these peaks diminished with decreasing levels of competition. The negative peaks appeared in all three iron treatments suggesting that the downregulation of pyoverdine production during the later phases of competition against BC is a general response to competition and not specific to iron limitation. Important to note is that peaks of pyoverdin levels appeared at earlier time points when more iron was supplemented, which is attributable to the accelerated growth observed with higher iron availabilities (Figure S2).

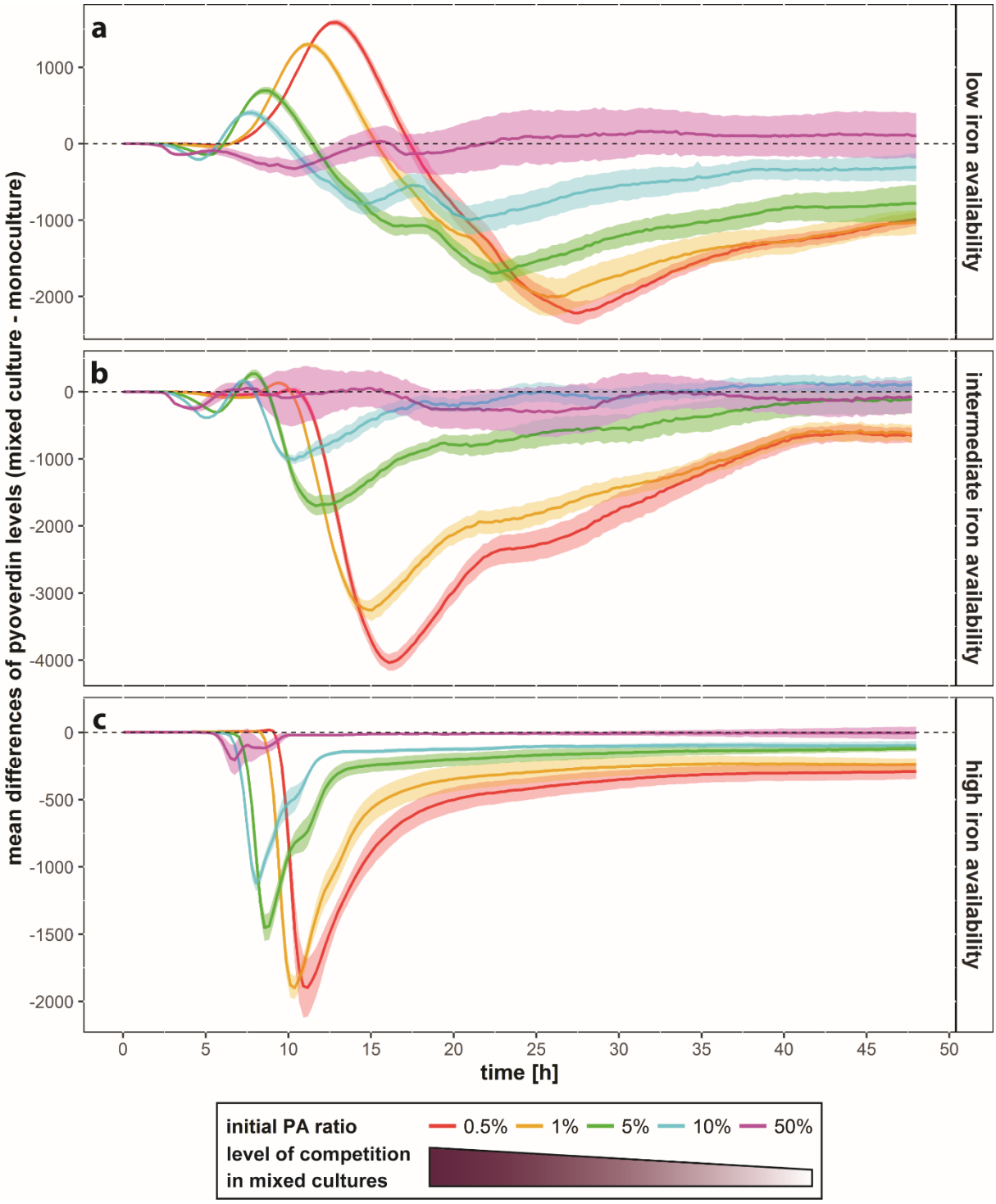


***Figure 2**: Temporal variation in pyoverdine levels of P. aeruginosa (PA), shown as difference in mixed cultures with B. cenocepacia (BC) compared to monocultures, in media with low **(a)**, intermediate **(b)** and high **(c)** iron availability. Positive and negative peaks indicate higher pyoverdine levels in mixed and monocultures, respectively. Positive peaks during the early phase of competition only appeared when iron availability was low **(a)**, suggesting that the upregulation of pyoverdine production is a direct response to inter-specific iron competition. Negative peaks at later phases of competition appeared across all iron treatments, suggesting that they reflect more general responses to competition. The amplitudes of both positive and negative peaks were fine-tuned in response to the level of inter-specific competition (illustrated by the purple bar). Pyoverdine fluorescence was measured over 48 hours and the mean differences of pyoverdine fluorescence of PA growing in mixed versus monoculture were calculated for each treatment and time point. The bold colored lines represent mean differences (7 to 8 replicates per treatment), whereas the faint colored areas show the 95% confidence intervals based on jackknifing (no overlap of the confidence intervals indicate significant differences between treatments).*

Temporal variation in pyoverdine production correlates with fitness parameters

Next, we examined how the fitness of PA was affected by our treatments. We found that the growth trajectories (Figure S2) and the different fitness parameters (initial phase (IP), growth rate and growth peak; Figure 3) mirrored the temporal variation in pyoverdine levels across treatments (Figure 2). Under iron-limited conditions, the initial phase (where growth rate equals zero) is significantly shorter in mixed compared to monocultures with the effect being most prominent under strong competition (LM: significant interaction between culture treatment and initial PA ratio, $F_{1,76}$=146.8, p<0.0001; Figure 3a). The trend was also apparent at intermediate iron availabilities ($F_{1,75}$=4.3, p=0.041; Figure 3b) but disappeared under high iron availabilities ($F_{1,66}$=1.8, p=0.19; Figure 3c). These data show that the increased availability of pyoverdine during the early phase of competition against BC in iron-limited media (Figure 2a) correlates with an earlier initiation of PA growth in mixed compared to monocultures.

This fitness advantage observed at the early phase of competition turned into a fitness disadvantage during later stages of competition, where the growth rate (Figure 3d-f) and growth yield (Figure 3g-i) of PA were reduced in mixed compared to monocultures. In all iron treatments, fitness values between mixed and monocultures differed most clearly when competition was strong, but converged when the level of competition was reduced (significant interaction between culture treatment and initial PA ratio: LM for the growth rate in media with limited iron, $F_{1,76}$=10.1, p= 0.002; intermediate iron, $F_{1,75}$=240.8, p<0.0001; and high iron, $F_{1,66}$=32.2, p<0.0001 – LM for the growth yield in media with limited iron: $F_{1,76}$=9.8, p=0.002; intermediate iron: $F_{1,75}$=20.6, p<0.0001; and high iron: $F_{1,66}$=8.8, p<0.004). These data show that the decreased availability of pyoverdine during the later stages of competition with BC correlates with reduced growth rate and yield of PA in mixed compared to monocultures.

What happened to BC during these competitions? Comparable to previous results, we found that BC is a relatively weak competitor, which is consistently outcompeted by PA regardless of the mixing ratio and the iron availability (Leinweber et al. 2017 and Figure S3). The low relative fitness of BC suggests that this species grows poorly in co-culture with PA. This indicates that the fitness effects we observed for PA are likely a direct consequence of competition sensing and the resulting response, and not an indirect effect due to BC consuming significant amounts of the available nutrients, thereby compromising the growth of PA.

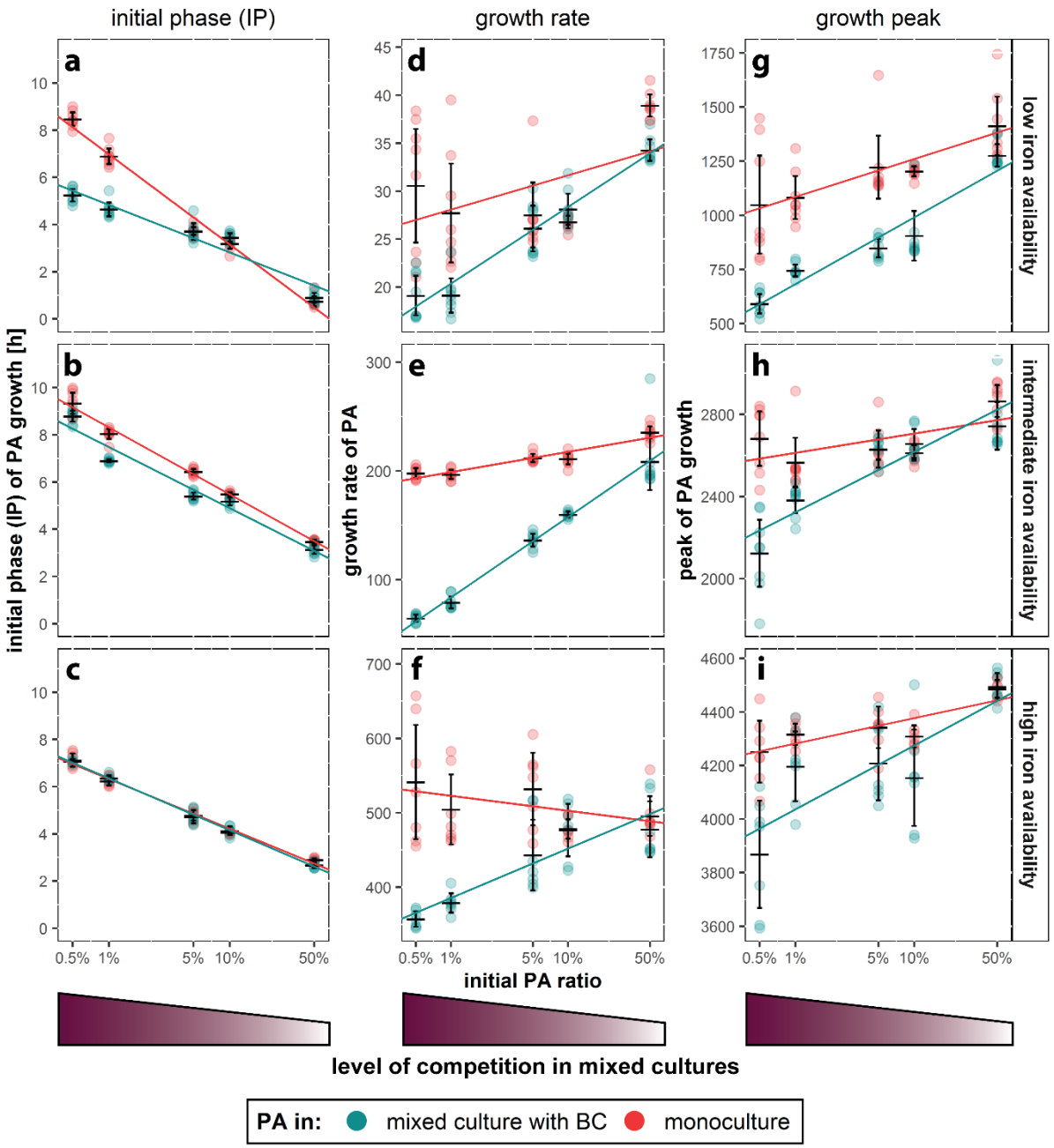


Figure 3: *Growth parameters of P. aeruginosa (PA) in mixed cultures with B. cenocepacia (BC, blue circles) and in monocultures (red circles). Growth parameters include the initial phase (IP, growth rate = 0) (a-c), maximal growth rate (d-f), and growth peak (g-i), measured in media with low (a, d, g), intermediate (b, e, h) and high (c, f, i) iron availability. Compared to monocultures, the IP was significantly shortened with high levels of competition under low iron availability (a), suggesting that an earlier onset of growth is a beneficial response to inter-specific iron competition. In contrast, the maximal growth rate and growth peak were significantly reduced in mixed cultures with high levels of competition across all iron treatments, suggesting that competition has some general negative fitness consequences for PA. Colored symbols represent single data points of 7 to 8 replicates per treatment, whereas black symbols show the mean ± 95% confidence interval. Colored lines depict the fits of the respective linear regressions.*

PA actively upregulates pyoverdine synthesis in response to competition

The strong correlation between the changes in pyoverdine profiles in response to competition and the fitness effects makes it hard to draw causalities between the two variables. Do bacteria indeed upregulate pyoverdine production in response to strong inter-specific iron competition, which in turn stimulates a faster initiation of growth? Or could it be that PA simply starts proliferating faster in response to competition? Faster growth would lead to higher cell numbers and thus to more available pyoverdine at the population level, without the individuals themselves changing their level of pyoverdine synthesis. In order to disentangle between these two scenarios, we examined the expression of the *pvdA* gene, encoding an enzyme involved in pyoverdine synthesis (Visca et al. 1994; Leoni et al. 1996), at the single cell level in mixed versus monocultures. We found, that under strong inter-specific competition for iron, *pvdA* expression was significantly increased in mixed compared to PA monocultures during the early competition phase (Figure 4; *t*-test between mixed and monoculture in iron limited medium at 10h after inoculation: $t_{480} = 12.86$, $p < 0.0001$; after 12h: $t_{269} = 6.52$, $p < 0.0001$; after 14h: $t_{782} = 12.27$, $p < 0.0001$). At later stages of competition, meanwhile, the pattern flipped and the *pvdA* expression was significantly reduced in mixed compared to monocultures (Figure 4; *t*-test at 20h after inoculation: $t_{325} = -6.7$, $p < 0.0001$). These findings provide strong support for the first scenario, where inter-specific competition for iron indeed stimulates increased pyoverdine synthesis during the early competition phase in conditions where siderophores are essential for iron scavenging, which then results in a fitness advantage for PA, in the form of a significantly reduced initial phase (Figure 3a).

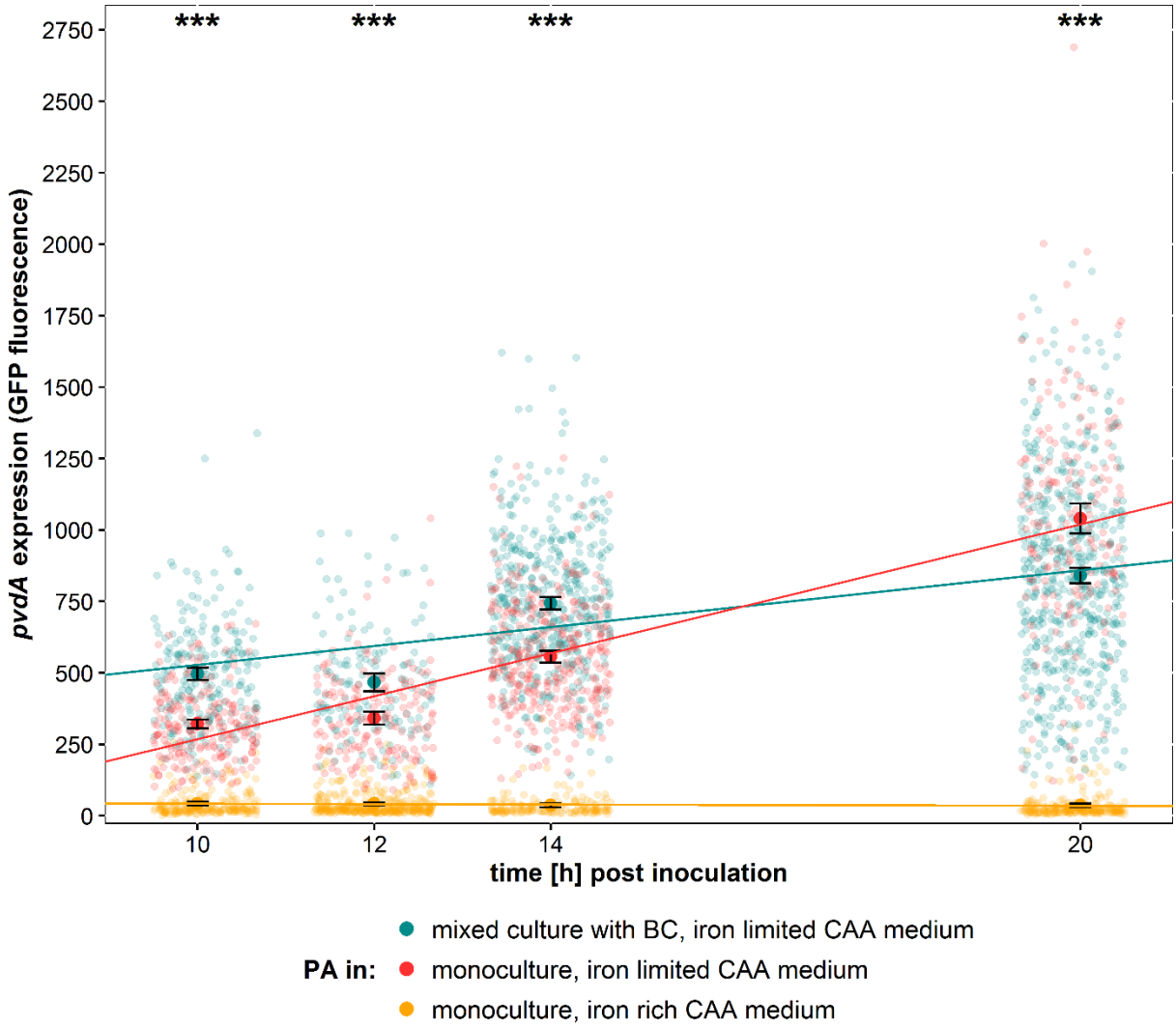


Figure 4: *Temporal variation in single-cell pvdA-GFP gene expression in P. aeruginosa (PA) when growing either in mixed cultures with B. cenocepacia (BC) (blue circles) or in monocultures (red circles) under low iron availability. During the early growth phase, the expression of pvdA (encoding an enzyme involved in pyoverdine synthesis) was significantly upregulated in mixed compared to monocultures. At a later growth phase, the pvdA expression levels switched, such that pvdA expression was significantly higher in mono- compared to mixed cultures. These results confirm that PA cells indeed upregulate pyoverdine synthesis in response to inter-specific iron competition during the early phase of competition. Under iron-rich conditions (yellow circles), there was very low pvdA expression, demonstrating the functionality and sensitivity of our GFP-reporter. While faint colored circles show GFP fluorescence values of single cells (n = 144 to 642 per treatment and time point), bold circles and error bars show means and 95% confidence intervals, respectively. Colored lines depict the fits of the respective linear regressions. For this experiment, PA was exposed to intense inter-specific competition (0.5% PA with 99.5% BC). Asterisks indicate significant (***P<0.0001) differences in pvdA expression between PA in mono- and mixed cultures in iron limited CAA medium.*

Discussion

It has long been recognized that siderophores are important modulators of intra-specific competition, as mutants that are defective for siderophore synthesis can exploit the siderophores produced by others, thereby compromising the fitness of producers and the community as a whole (Griffin et al. 2004; West et al. 2006; Ross-Gillespie et al. 2007, 2015; Kümmerli et al. 2009a; Scholz and Greenberg 2015). Here, we show that siderophores are also important factors in inter-specific competition. In

particular, we found that: (a) the bacterium *P. aeruginosa* (PA) up-regulates the production of its primary siderophore, pyoverdine, during the early phase of competition against *B. cenocepacia* (BC); (b) this phenotypic plastic upregulation was fine-tuned in response to the level of competition; and (c) the early upregulation of pyoverdine confers a fitness benefit in the form of an earlier initiation of growth in mixed compared to monocultures. Our findings suggest that PA cannot only sense the presence of BC, but also the level of competition imposed by this competitor, and gradually adjust its siderophore response to match prevailing conditions. Our results support the idea that the primary goal of siderophores in inter-specific competition is to access and bind iron before the competitor does so (Fgaier and Eberl 2010, 2011; Schiessl et al. 2016; Leinweber et al. 2017; Niehus et al. 2017). This strategy locks iron away from the competitor, and reserves it for strain members possessing the matching receptor for uptake – a strategy that can be particularly successful when siderophore production is increased in response to competition. More generally, our findings indicate that phenotypic plasticity in response to competition might be a common feature in microorganisms.

Our results raise the question how can PA sense the level of inter-specific competition in such a fine-tuned manner (Cornforth and Foster 2013)? We can offer three mutually non-exclusive explanations for this phenomenon. First, PA could sense the presence of BC via indirect cues, such as increased resource competition. Although we stated before that BC probably does not inhibit PA's growth by consuming high amounts of nutrients, because PA strongly suppresses BC in competition (Leinweber et al. 2017 and Figure S3), it is still plausible that BC could block PA from accessing essential nutrients. Under low and intermediate iron availabilities, a realistic scenario is that BC secretes its own siderophore ornibactin (Tyrrell et al. 2015), which binds iron and thus reduces iron availability for PA. In response, PA could then upregulate pyoverdin synthesis (Weaver and Kolter 2004). Previous work showed that PA indeed possesses the regulatory elements for fine-tuned adjustments: a positive signaling system, telling the cell when more pyoverdine is needed, interacts with a negative feedback loop, curbing pyoverdine production when intra-cellular iron stocks become replenished (Lamont et al. 2002; Tiburzi et al. 2008). The interaction between the two regulatory elements then determines the adequate level of pyoverdine production, with the system being sensitive to both abiotic (e.g. how strongly iron is bound in the medium) and biotic (e.g. the presence of non-producers serving as iron sinks) factors, which affect the relative iron availability (Kümmerli et al. 2009b; Dumas et al. 2013; Harrison 2013). Second, PA could directly sense diffusible molecules secreted by BC and use those as cues to mount a specific response. Candidates for such diffusible cues are quorum sensing molecules, extra-cellular enzymes, or toxins (Jimenez et al. 2012). These molecules could be detected via specific receptors (i.e. a phenomenon called 'cross-talk' for quorum sensing molecules (Williams 2007)) or through their negative effects. For example, diffusible molecules such as extra-cellular enzymes or toxins can induce cell wall damage, DNA damage, or oxidative stress (Cornforth and Foster 2013),

which could trigger responses as those observed in our study. Finally, competition sensing could also occur through direct cell-to-cell contact, whereby structural elements of the cell envelop could serve as cues to mount responses (Hood et al. 2010; Silverman et al. 2012; Basler et al. 2013; Ruhe et al. 2013; Mercy et al. 2016; McNally et al. 2017).

While the above considerations elucidate the early phase of competition between PA and BC, which is characterized by increased pyoverdine production and the accelerated initiation of growth under strong iron limitation, the patterns of pyoverdine availability and fitness change fundamentally at the later stages of competition. Here, pyoverdine levels declined in mixed compared to monocultures (Figure 2), and PA growth was significantly slowed down (Figure 3d-i). While these effects are independent of the iron availability in the medium, they clearly reflect a response to competition, because the magnitude of the responses significantly correlated with the level of competition. What type of general response could this be? One obvious explanation is that nutrients became more quickly exhausted in our mixed cultures, because overall cell density at the start was higher in mixed compared to the monocultures (note that we kept PA cell density constant between mixed and mono cultures). However, two lines of evidence speak against this explanation. For one thing, it is questionable to what extent BC actually contributes to the overall nutrient consumption, as BC is strongly outcompeted by PA at all iron concentrations (Leinweber et al. 2017 and Figure S3). Furthermore, PA exhibited reduced growth rates in mixed cultures long before nutrients became exhausted (Figures 3d-f and S2).

A more plausible explanation is that PA reacts at multiple fronts to the presence of BC, and does not solely increase pyoverdine production, the focal trait of our study. Indeed, previous work revealed that PA can increase biofilm formation, pyocyanin production and activate the type-6-secretion system when facing a competitor (Basler et al. 2013; Trejo-Hernandez et al. 2014; LeRoux et al. 2015; Oliveira et al. 2015). The activation or upregulation of this arsenal likely incurs significant metabolic costs, which are expected to impair growth rate, exactly as observed in our experiments (Figure 3d-i). Unlike siderophores, these additional responses should be mounted regardless of the level of iron availability, and costs are thus expected to manifest under all conditions tested in our study (Figure 3d-i). Taken together, our data point towards an evolutionary trade-off between the relative fitness advantage PA can gain, by mounting a rapid and effective response against its competitors, and the absolute fitness reduction PA experiences due to the costly anti-competitor response. More research integrating responses on multiple competitive traits is clearly required to test this hypothesis.

In conclusion, our study reveals that PA plastically adjusts the synthesis of its primary siderophore in response to the level of competition with BC, a response that had direct consequences for PA's fitness. Our study thus highlights that siderophores are important agents in inter-specific competition for iron. This contrasts with their role as growth-promoting public goods, which can be cooperatively shared

between members of the same strain. Both within-species cooperation and between-species competition seem to be important elements contributing to community assembly and composition. Accordingly, integrative studies focusing on both types of interactions in multi-species communities are required to better understand the relative importance of the two roles of siderophores.

Acknowledgements

We thank Dr. Sebastian Wenzel and Dr. Ruben Dezeure for helping with the data analysis and the Swiss National Science Foundation (grant no. PP00P3-139164) and European Research Council (grant no. 681295) for funding.

Literature

Agrawal, A. A. 2001. Phenotypic plasticity in the interactions and evolution of species. Science 294:321–326.

Bakkal, S., S. M. Robinson, C. L. Ordonez, D. A. Waltz, and M. A. Riley. 2010. Role of bacteriocins in mediating interactions of bacterial isolates taken from cystic fibrosis patients. Microbiology 156:2058–2067.

Basler, M., B. T. Ho, and J. J. Mekalanos. 2013. Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. Cell 152:884–894.

Becker, J., N. Eisenhauer, S. Scheu, and A. Jousset. 2012. Increasing antagonistic interactions cause bacterial communities to collapse at high diversity. Ecol. Lett. 15:468–474.

Boege, K. 2010. Induced responses to competition on multi-trait phenotypic plasticity. Ecology 91:2628–2637.

Cornforth, D. M., and K. R. Foster. 2013. Competition sensing: the social side of bacterial stress responses. Nat. Rev. Microbiol. 11:285–293.

Cortesi, F., W. E. Feeney, M. C. O. Ferrari, P. A. Waldie, G. A. C. Phillips, E. C. McClure, H. N. Sköld, W. Salzburger, N. J. Marshall, and K. L. Cheney. 2015. Phenotypic plasticity confers multiple fitness benefits to a mimic. Curr. Biol. 25:949–954.

Curtis, T. P., W. T. Sloan, and J. W. Scannell. 2002. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. U. S. A. 99:10494–10499.

Dumas, Z., A. Ross-Gillespie, and R. Kümmerli. 2013. Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. Proc. R. Soc. B 280:20131055.

Fgaier, H., and H. J. Eberl. 2010. A competition model between Pseudomonas fluorescens and pathogens via iron chelation. J. Theor. Biol. 263:566–578.

Fgaier, H., and H. J. Eberl. 2011. Antagonistic control of microbial pathogens under iron limitations by siderophore producing bacteria in a chemostat setup. J. Theor. Biol. 273:103–114.

Fordyce, J. A. 2006. The evolutionary consequences of ecological interactions mediated through phenotypic plasticity. J. Exp. Biol. 209:2377–2383.

Foster, K. R., and T. Bell. 2012. Competition, not cooperation, dominates interactions among culturable microbial species. Curr. Biol. 22:1845–1850.

Gotschlich, A., B. Huber, O. Geisenberger, A. Togl, A. Steidle, K. Riedel, P. Hill, B. Tummler, P. Vandamme, B. Middleton, et al. 2001. Synthesis of multiple N-acylhomoserine lactones is wide-spread among the members of the Burkholderia cepacia complex. Syst. Appl. Microbiol. 24:1–14.

Griffin, A., S. A. West, and A. Buckling. 2004. Cooperation and competition in pathogenic bacteria. Nature 430:1024–1027.

Harrison, F. 2013. Social versus nonsocial cues and responses: a reply to Alizon. J. Evol. Biol. 26:2297–2298.

Harrison, F., J. Paul, R. C. Massey, and A. Buckling. 2008. Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. ISME J. 2:49–55.

Hersman, L. E., A. Huang, P. A. Maurice, and J. E. Forsythe. 2000. Siderophore production and iron reduction by Pseudomonas mendocina in response to iron deprivation. Geomicrobiol. J. 17:261–273.

Hood, R. D., P. Singh, F. Hsu, T. Güvener, M. A. Carl, R. R. S. Trinidad, J. M. Silverman, B. B. Ohlson, K. G. Hicks, R. L. Plemel, et al. 2010. A type VI secretion system of Pseudomonas aeruginosa targets a toxin to bacteria. Cell Host Microbe 7:25–37.

Jimenez, P. N., G. Koch, J. A. Thompson, K. B. Xavier, R. H. Cool, and W. J. Quax. 2012. The multiple signaling systems regulating virulence in Pseudomonas aeruginosa. Microbiol. Mol. Biol. Rev. 76:46–65.

Joshi, F., G. Archana, and A. Desai. 2006. Siderophore cross-utilization amongst rhizospheric bacteria and the role of their differential affinities for Fe3+ on growth stimulation under iron-limited conditions. Curr. Microbiol. 53:141–147.

Kahm, M., G. Hasenbrink, H. Lichtenberg-Fraté, J. Ludwig, and M. Kschischo. 2010. grofit: Fitting Biological Growth Curves with R Matthias. J. Stat. Softw. 33.

Kaneko, Y., M. Thoendel, O. Olakanmi, B. E. Britigan, and P. K. Singh. 2007. The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. J. Clin. Invest. 117:877–888.

Kümmerli, R., A. S. Griffin, S. A. West, A. Buckling, F. Harrison, A. S. Griffin, S. A. West, A. Buckling, and F. Harrison. 2009a. Viscous medium promotes cooperation in the pathogenic bacterium Pseudomonas aeruginosa. Proc. R. Soc. B 276:3531–3538.

Kümmerli, R., N. Jiricny, L. S. Clarke, S. A. West, and A. S. Griffin. 2009b. Phenotypic plasticity of a cooperative behaviour in bacteria. J. Evol. Biol. 22:589–598.

Lamont, I. L., P. Beare, U. Ochsner, A. I. Vasil, and M. L. Vasil. 2002. Siderophore-mediated signaling regulates virulence factor production in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 99:7072–7077.

Leggett, H. C., S. P. Brown, and S. E. Reece. 2014. War and peace: social interactions in infections. Philos. Trans. R. Soc. B 369:20130365.

Leinweber, A., R. Fredrik Inglis, and R. Kümmerli. 2017. Cheating fosters species co-existence in well-mixed bacterial communities. ISME J. 11:1179–1188.

Leoni, L., A. Ciervo, N. Orsi, and P. Visca. 1996. Iron-regulated transcription of the pvdA gene in Pseudomonas aeruginosa: effect of Fur and PvdS on promoter activity. J. Bacteriol. 178:2299–2313.

LeRoux, M., R. L. Kirkpatrick, E. I. Montauti, B. Q. Tran, S. B. Peterson, B. N. Harding, J. C. Whitney, A. B. Russell, B. Traxler, Y. A. Goo, et al. 2015. Kin cell lysis is a danger signal that activates antibacterial pathways of Pseudomonas aeruginosa. Elife 4:1–25.

McNally, L., E. Bernardy, J. Thomas, A. Kalziqi, J. Pentz, S. P. Brown, B. K. Hammer, P. J. Yunker, and W. C. Ratcliff. 2017. Killing by Type VI secretion drives genetic phase separation and correlates with increased cooperation. Nat. Commun. 8:14371.

Mercy, C., B. Ize, S. P. Salcedo, S. de Bentzmann, and S. Bigot. 2016. Functional Characterization of Pseudomonas Contact Dependent Growth Inhibition (CDI) Systems. PLoS One 11:e0147435.

Miethke, M., and M. A. Marahiel. 2007. Siderophore-based iron acquisition and pathogen control. Microbiol. Mol. Biol. Rev. 71:413–451.

Niehus, R., A. Picot, N. M. Oliveira, S. Mitri, and K. R. Foster. 2017. The evolution of siderophore production as a competitive trait. Evolution 71:1443–1455.

Oliveira, N. M., E. Martinez-Garcia, J. Xavier, W. M. Durham, R. Kolter, W. Kim, and K. R. Foster. 2015. Biofilm formation as a response to ecological competition. PLoS Biol. 13.

Prats, C., A. Giró, J. Ferrer, D. López, and J. Vives-Rego. 2008. Analysis and IbM simulation of the stages in bacterial lag phase: Basis for an updated definition. J. Theor. Biol. 252:56–68.

R Development Core Team. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ratledge, C., and L. G. Dover. 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. 54:881–941.

Rinke, C., P. Schwientek, A. Sczyrba, N. N. Ivanova, I. J. Anderson, J.-F. Cheng, A. Darling, S. Malfatti, B. K. Swan, E. A. Gies, et al. 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature 499:431–437.

Ross-Gillespie, A., Z. Dumas, and R. Kümmerli. 2015. Evolutionary dynamics of interlinked public goods traits: an experimental study of siderophore production in Pseudomonas aeruginosa. J. Evol. Biol. 28:29–39.

Ross-Gillespie, A., A. Gardner, S. A. West, and A. S. Griffin. 2007. Frequency Dependence and Cooperation : Theory and a Test with Bacteria. Am. Nat. 170:331–342.

Ruhe, Z. C., D. A. Low, and C. S. Hayes. 2013. Bacterial contact-dependent growth inhibition. Trends Microbiol. 21:230–237.

S original from StatLib and by Rob Tibshirani. R port by Friedrich Leisch. 2015. Bootstrap: Functions for the book "An Introduction to the Bootstrap". R package version 2015.2.

Schiessl, K., E. M.-L. Janssen, S. M. Kraemer, K. McNeill, and M. Ackermann. 2016. Magnitude and mechanism of siderophore-mediated competition in bacterial interactions. bioRxiv.

Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, et al. 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9:676–682.

Scholz, R. L., and E. P. Greenberg. 2015. Sociality in Escherichia coli: Enterochelin is a private good at low cell density and can be shared at high cell density. J. Bacteriol. 197:2122–2128.

Silverman, J. M., Y. R. Brunet, E. Cascales, and J. D. Mougous. 2012. Structure and regulation of the type VI secretion system. Annu. Rev. Microbiol. 66:453–72.

Sommer, C., C. Strähle, U. Köthe, and F. A. Hamprecht. 2011. ilastik: Interactive Learning and Segmentation Toolkit. Eighth IEEE Int. Symp. Biomed. Imaging 230–233.

Tashiro, Y., Y. Yawata, M. Toyofuku, H. Uchiyama, and N. Nomura. 2013. Interspecies interaction between Pseudomonas aeruginosa and other microorganisms. Microbes Environ. 28:13–24.

Tiburzi, F., F. Imperi, and P. Visca. 2008. Intracellular levels and activity of PvdS, the major iron starvation sigma factor of Pseudomonas aeruginosa. Mol. Microbiol. 67:213–227.

Traxler, M. F., J. D. Watrous, T. Alexandrov, P. C. Dorrestein, and R. Kolter. 2013. Interspecies interactions stimulate diversification of the Streptomyces coelicolor secreted metabolome. MBio 4:e00459-13.

Trejo-Hernandez, A., A. Andrade-Dominguez, M. Hernandez, and S. Encarnacion. 2014. Interspecies competition triggers virulence and mutability in Candida albicans-Pseudomonas aeruginosa mixed biofilms. Isme J. 1974–1988.

Tyrrell, J., and M. Callaghan. 2015. Iron acquisition in the Cystic Fibrosis lung and potential for novel therapeutic strategies. Microbiology 162:191–205.

Tyrrell, J., N. Whelan, C. Wright, I. Sá-Correia, S. McClean, M. Thomas, and M. Callaghan. 2015. Investigation of the multifaceted iron acquisition strategies of Burkholderia cenocepacia. Biometals 28:367–380.

Visca, P., A. Ciervo, and N. Orsi. 1994. Cloning and nucleotide sequence of the pvdA gene encoding the pyoverdine biosynthetic enzyme L-ornithine N5-oxygenase in Pseudomonas aeruginosa. J. Bacteriol. 176:1128–1140.

Weaver, V. B., and R. Kolter. 2004. Burkholderia spp. alter Pseudomonas aeruginosa physiology through iron sequestration. J Bacteriol 186:2376–2384.

Weigert, M., and R. Kümmerli. 2017. The physical boundaries of public goods cooperation between surface-attached bacterial cells. Proc. R. Soc. B in press.

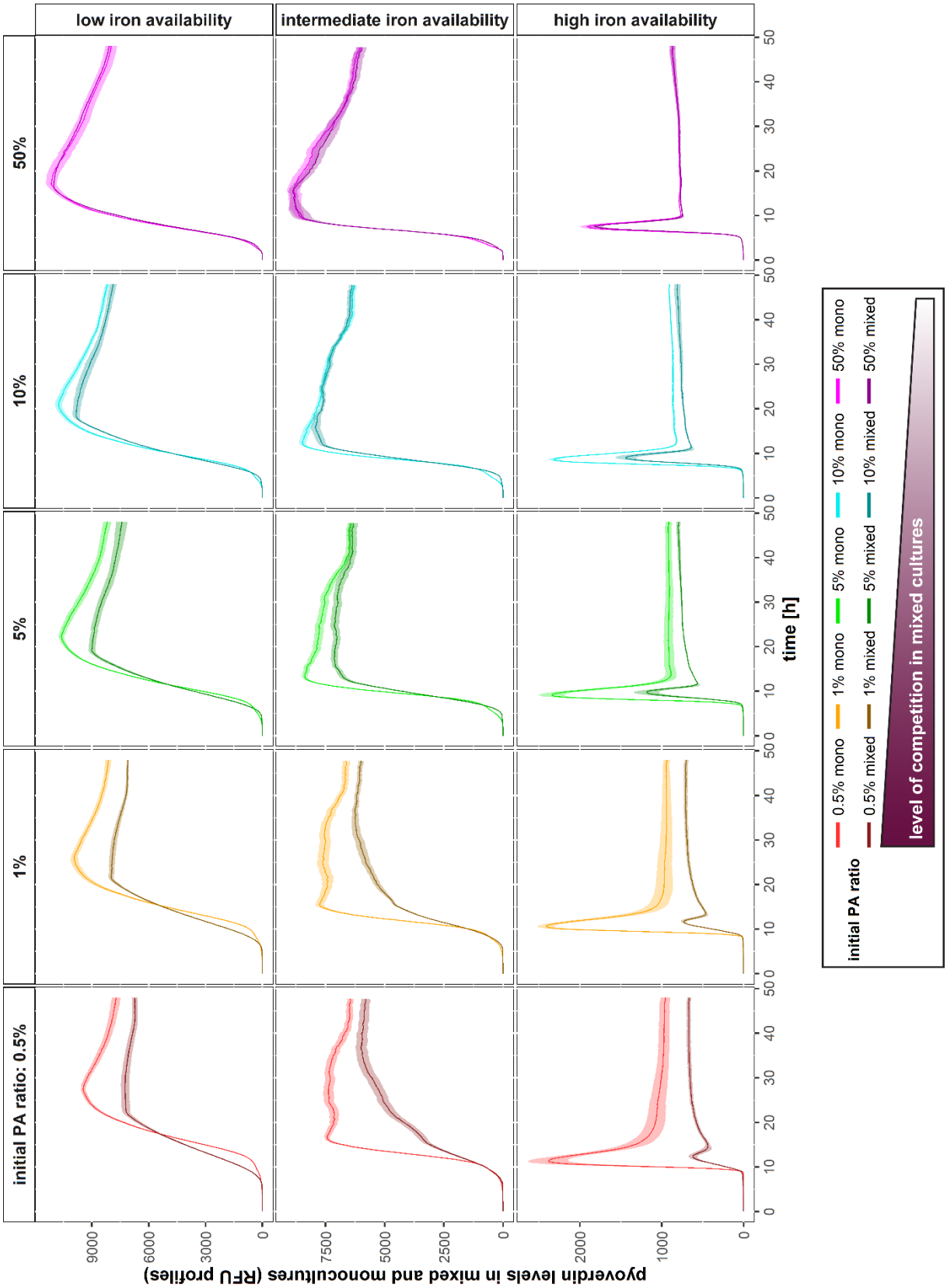
West-Eberhard, M. J. 1989. Phenotypic Plasticity and the origins of diversity. Annu. Rev. Ecol. Evol. Syst. 20:249–278.

West, S. A., A. S. Griffin, A. Gardner, and S. P. Diggle. 2006. Social evolution theory for microorganisms. Nat Rev Microbiol 4:597–607.

Whitman, W. B., D. C. Coleman, and W. J. Wiebe. 1998. Prokaryotes: The unseen majority. Proc. Natl. Acad. Sci. U. S. A. 95:6578–6583.

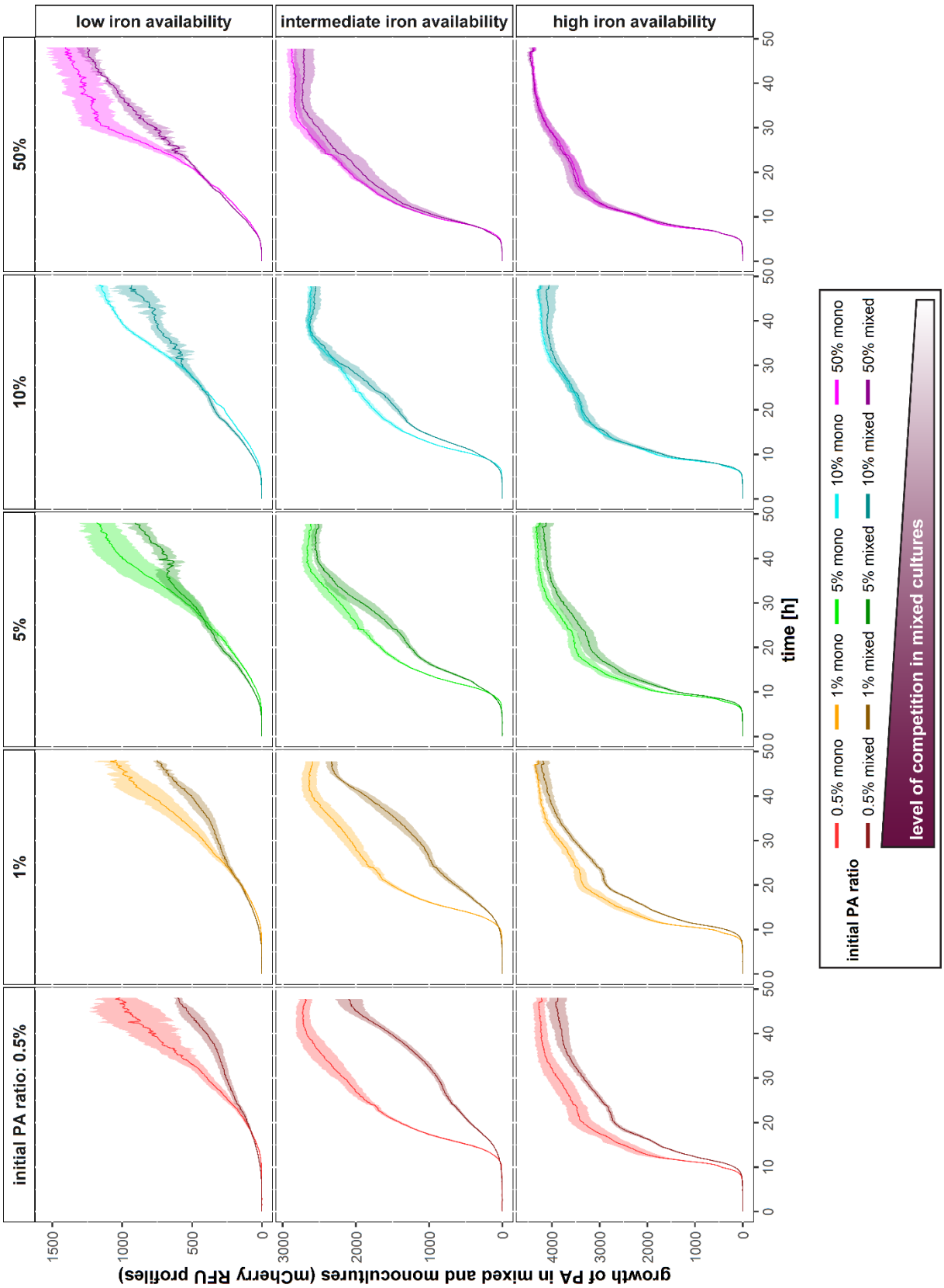
Williams, P. 2007. Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiology 153:3923–3938.

4.1. Supporting information



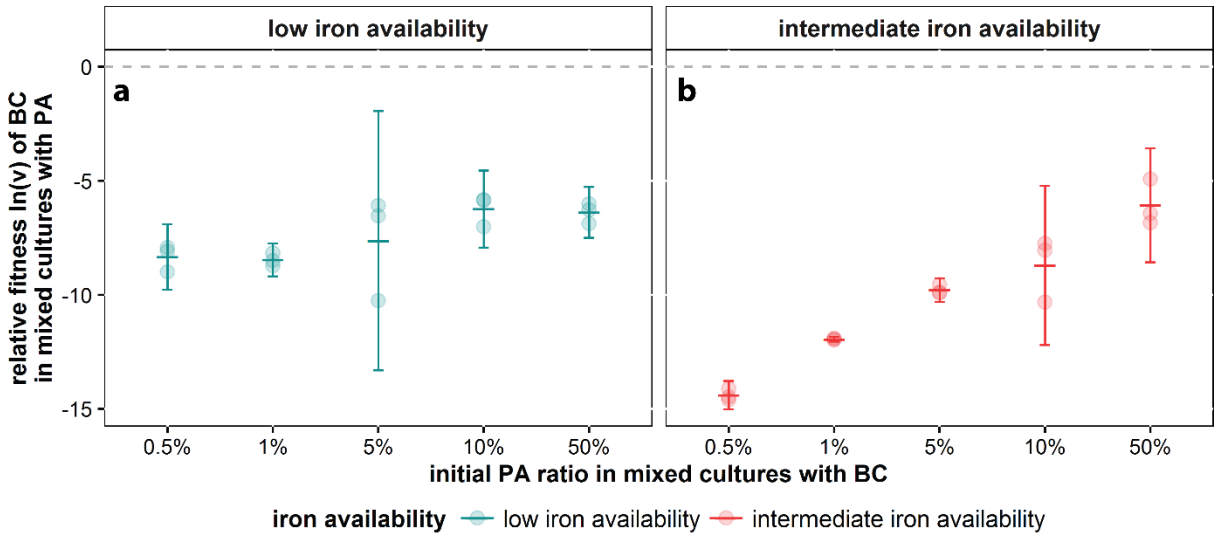
Supporting Figure 1: *Pyoverdine profiles of P. aeruginosa (PA) grown in mixed cultures with B. cenocepacia (BC) and in monocultures in CAA medium with low, intermediate or high iron availability over 48 hours. Pyoverdine levels are higher during the early competition phase, but only under severe competitive pressure from BC and under strong iron limitation, suggesting that increased pyoverdine levels are a specific response of PA to iron competition with BC. In contrast, later pyoverdine levels*

are reduced in mixed compared to monocultures in all iron treatments, suggesting this to be a more general response to inter-specific competition. Moreover, PA can fine-tune the pyoverdine levels in response to varying levels of competition (illustrated by the purple bar), in a way that pyoverdine levels converge in mixed and monocultures, when the level of competition declines. Across all levels of competition, the pyoverdine levels decrease when more iron is readily available. To investigate the effect of different levels of competition on pyoverdine, we varied the initial ratio of BC in the mixed cultures (50-99.5%) and inoculated respective PA monocultures with the same initial PA cell density (BC was substituted with an equal volume of 0.8% NaCl). Bacteria were cultured under three different iron regiments (low iron availability = CAA + 100 µg/ml transferrin; intermediate iron availability = CAA + 100 µg/ml transferrin + 0.5uM $FeCl_3$; high iron availability = CAA + 20uM $FeCl_3$). We then measured the autofluorescence of pyoverdine (excitation = 400 nm, emission = 460 nm) over 48 hours of 7 to 8 replicates per treatment. Light and dark colors represent pyoverdine fluorescence in PA monocultures and in PA and BC mixed cultures, respectively. Solid lines show the mean fluorescence over the replicates of one treatment and the faint areas depict the 95% confidence interval.



Supporting Figure 2: *Growth of P. aeruginosa (PA) in either mixed cultures with B. cenocepacia (BC) or in monocultures in CAA medium with low, intermediate or high iron availability over 48 hours. Under strong competition and low iron availability, PA shows an earlier initiation of growth in mixed cultures with BC compared to the respective monocultures, suggesting a fitness advantage of PA over BC in the competition for iron. This effect disappears with higher iron availabilities. Later growth of PA is compromised by strong competition under all iron concentrations compared to respective monocultures, indicating a generally negative effect of inter-specific competition on the fitness of PA. PA's growth trajectories in mixed cultures approach*

the growth trajectories of PA monocultures when the level of competition decreases (illustrated by the purple bar). Thus, PA's growth phenotype gradually changes in response to different levels of competition. Additionally, PA's growth fitness increases across all levels of competition, when iron becomes more available. We manipulated the level of BC competition on PA by varying the initial ratio of BC in mixed cultures (50-99.5%) and inoculated respective PA monocultures with the same initial PA cell density (BC was substituted with an equal volume of 0.8% NaCl). Bacteria were cultured under different iron regiments (low iron availability = CAA + 100 µg/ml transferrin; intermediate iron availability = CAA + 100 µg/ml transferrin + 0.5uM $FeCl_3$; high iron availability = CAA + 20uM $FeCl_3$). We then measured growth of PA only, by following the mCherry fluorescence of the PA strain (excitation = 582 nm, emission = 620 nm). The BC strain did not carry a fluorescent marker. Light and dark colors represent PA's growth in monocultures and mixed cultures, respectively. Solid lines show the mean mCherry fluorescence of 7 to 8 replicates of one treatment and the faint areas depict the 95% confidence interval.



Supporting Figure 3: *Relative fitness ln(v) of B. cenocepacia (BC) in pairwise competitions with P. aeruginosa (PA) in CAA medium with limited **(a)** and intermediate **(b)** iron availability across different initial PA ratios. PA significantly outcompeted BC under all conditions. The relative fitness v of BC was calculated following the protocol described in (Leinweber et al. 2017). In short, we determined the initial and final frequencies of PA and BC before and after measuring pyoverdin levels and PA growth for 48 hours, by plating a fraction of the mixed cultures (see Table S2 for more details) on LB agar plates containing 20 µM $FeCl_3$. After incubating the plates overnight at 37°C and an additional 24 hours at room temperature to ensure the maturation of the fluorescent marker, we counted the colony forming units (CFU) of PA and BC using an imaging platform. Colonies were distinguished based on mCherry fluorescence of PA. We then calculated the relative fitness of BC with the formula: $v=[a_t \times (1-a_0)]/[a_0 \times (1-a_t)]$, where a_0 and a_t are the initial and final frequency of BC, respectively (Ross-Gillespie et al. 2007). Faint colored circles show single data points, bold horizontal lines and error bars show the mean and the 95% confidence interval, respectively, of 3 replicates.*

***Supplementary Table 1**: Dilutions of cultures and number of analyzed PA cells for the single cell pvdA gene expression analysis per treatment and time point.*

Time point	Culture	Medium	Analyzed dilution	Number of analyzed cells
10 h	Mixed culture	iron limited CAA medium	10^{-1}	272
	Monoculture	iron limited CAA medium	10^{-1}	253
	Monoculture	iron rich CAA medium	10^{-2}	235
12 h	Mixed culture	iron limited CAA medium	10^{-1}	144
	Monoculture	iron limited CAA medium	10^{-1}	177
	Monoculture	iron rich CAA medium	10^{-2}	325
14 h	Mixed culture	iron limited CAA medium	10^{-2}	470
	Monoculture	iron limited CAA medium	10^{-1}	330
	Monoculture	iron rich CAA medium	10^{-3}	154
20 h	Mixed culture	iron limited CAA medium	10^{-1}	642
	Monoculture	iron limited CAA medium	10^{-2}	207
	Monoculture	iron rich CAA medium	10^{-3}	251

References

Leinweber, A., R. Fredrik Inglis, and R. Kümmerli. 2017. Cheating fosters species co-existence in well-mixed bacterial communities. ISME J. 11:1179–1188.

Ross-Gillespie, A., A. Gardner, S. A. West, and A. S. Griffin. 2007. Frequency Dependence and Cooperation : Theory and a Test with Bacteria. Am. Nat. 170:331–342.

5. Third Project: The bacterium *Pseudomonas aeruginosa* senses and responses to diffusible cues of its competitor *Burkholderia cenocepacia*

This project was funded by the URPP evolution in action grant for innovative pilot projects (2016).

Authors: **Anne Leinweber[1], Stefan Wyder[1,2], Rolf Kümmerli[1]**

[1] Department of Plant and Microbial Biology, University of Zurich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

[2] Institute of Molecular Life Sciences, University of Zurich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

Abstract

Bacteria are ubiquitous organisms that naturally live in polymicrobial communities, where competition for resources and space is high. How bacteria sense inter-specific competition and mount a respective competitive response is not yet well understood. Here, we used an RNA-sequencing approach to unravel how the opportunistic human pathogen *Pseudomonas aeruginosa* (PA) reacts to its naturally co-occurring competitor *Burkholderia cenocepacia* (BC). To find out if diffusible cues transmit the presence of the competing species BC to PA, we grew PA in fresh medium mixed with BC spent culture supernatant, and compared its gene expression profile with two control conditions: PA grown in fresh medium mixed with its own supernatant and fresh medium only. We assembled an *a priori* list of PA traits, which are known to mediate competition of PA with other species, from the literature, to investigate if PA initiates a defensive and/or an attacking response when sensing BC cues. Our RNA sequencing approach revealed that PA fundamentally changed its global gene expression pattern in response to BC-derived diffusible cues, mounting a complex multivariate response. Iron availability of the medium strongly modulated this response. Under iron limitation, PA mainly initiates an attacking response by increasing the expression of genes coding for the Lipase A and for the synthesis of the highly toxic compounds hydrogen cyanide and phenazines. Under iron repletion, PA's response to BC cues is broader, involving increased expression of genes encoding the Lipoxygenase LoxA, a putative protease ImpA (PA0572), motility (flagella assembly), iron uptake (pyoverdine synthesis), pyocins, and the type-6-secretion system with its effector proteins. The expression of HHQ synthesis genes (quorum sensing precursor molecule with antibacterial properties) was upregulated under high and low iron availability alike. This study demonstrates that bacteria can use diffusible cues from competitors to initiate multivariate competitive responses and that environmental factors can have profound effects on the composition of this response.

Introduction

Sequencing technologies have revealed that natural bacterial communities are incredibly diverse, and that myriads of taxa can live in close proximity to one another (Curtis et al. 2002; Rusch et al. 2007; The Human Microbiome Project 2012; Rinke et al. 2013; Sunagawa et al. 2015). From an ecological perspective, the living together of completely different species raises some interesting issues. For one thing, coexistence could promote intense inter-specific competition for essential resources such as nutrients, space and oxygen (Hibbing et al. 2010; Stubbendieck and Straight 2016; Stubbendieck et al. 2016). On the other hand, one could also assume that the level of inter-specific competition fluctuates both spatially and temporarily, and that bacteria might have evolved ways to sense the prevailing level of inter-specific competition in order to mount adequate responses (Cornforth and Foster 2013; Westhoff et al. 2017). Although there are many studies examining multi-species communities, we know surprisingly little about how bacteria react to competitors. Open questions include: (i) whether bacteria can sense diffusible cues and initiate a competitive response before they actually encounter competitors; (ii) whether responses depend on abiotic factors of the environment such as nutrient availability; (iii) whether bacteria mount a few specific responses or react more globally at multiple fronts; and (iv) whether bacteria invest into defense and/or attack mechanisms.

Here, we tackle these questions by examining the global responses at the transcriptome level of the bacterium *Pseudomonas aeruginosa* (PA) when exposed to diffusible cues of its competitor *Burkholderia cenocepacia* (BC). We chose these two bacteria as a model system because both species are opportunistic human pathogens, which can co-infect lungs of cystic fibrosis patients, but can also potentially co-exist in natural soil habitats (Coenye and Vandamme 2003; Weaver and Kolter 2004; Harrison 2007; Fang et al. 2011; Aujoulat et al. 2012; Maravic et al. 2012; Suzuki et al. 2013; Hariprasad et al. 2014; Nair et al. 2015; Schaffer 2015). In addition, laboratory experiments revealed conditions where the two species can co-exist in mixed biofilms, in mouse infection models (Riedel et al. 2001), and even in planktonic cultures (Leinweber et al. 2017a), although PA strongly dominates BC in the latter scenario. Given this relevant biological background, we reasoned that PA might have evolved ways to sense BC and mount adequate competitive responses.

To be able to address the outlined questions, we quantified the transcriptome of PA under six different growth conditions in three independent biological replicates per treatment using RNA sequencing. To test whether PA responds to diffusible cues secreted by BC into the medium, we grew PA in 70 % fresh media either supplemented with 30% supernatant from BC or its own supernatant. As a control, we further grew PA in fresh media without supernatant. To test whether responses depend on nutrient availability we measured the transcriptome for all three conditions outlined above, both under nutrient rich and under conditions where bacterial growth is significantly compromised by low iron

availability. Our global transcriptomics approach was further suitable to assess, whether bacteria mount a few specific responses or react in a multivariate way to competition, and whether these responses include defense and/or attack mechanisms.

Prior to data analysis, we conducted an extensive literature search to identify traits of PA that could potentially be involved in interspecific competition. In this context, our transcriptome analysis differs from traditional approaches, which typically assemble a list of the most differentially regulated genes between two treatments, followed by *post-hoc* interpretations. We in contrast assembled *a priori* a list of traits, potentially involved in inter-specific competition. This allowed us to examine which of these candidate traits were significantly up-regulated in response to competition, and which ones were not. As outlined above, we were interested in whether PA invests in both defense and attack strategies when sensing competitors. Classic defensive traits could involve protective biofilm formation or motility to move away from competitors as has been shown in several laboratory studies with bacterial species (Mah and O'Toole 2001; Hall-Stoodley et al. 2004; Hoffman et al. 2005; Kaiser 2007; Marr et al. 2007; Hibbing et al. 2010; Graff et al. 2013; Oliveira et al. 2015). In our analysis, we thus focused on genes encoding compounds/structures involved in adhesion (adhesins, lectins, fimbriae, pili), biofilm matrix formation (exo-polysaccharides) and motility (biosurfactants, pili, flagella). Meanwhile, attack strategies are known to involve enzymes or toxins either secreted into the medium or injected directly into target cells via secretion systems upon cell-to-cell contact (Bakkal et al. 2010; Bleves et al. 2010; Pierson and Pierson 2010; Filloux 2011; Tashiro et al. 2013; Chen et al. 2015; Bernier et al. 2016; Mercy et al. 2016; Sana et al. 2016). Accordingly, we examined expression levels of genes either directly encoding such interference molecules, or genes coding for enzymes and structures required for the synthesis and translocation of such compounds. Specifically, we focused on secreted lipolytic and proteolity enzymes, phosphatases, phenazines, pyocins, hydrogen cyanide, other effector proteins, and the various secretion systems required for toxin delivery. Because many of the traits we categorized here as being either of defensive or attacking nature are controlled by quorum sensing (QS) in a cell-density dependent manner, we also tested for the differential expression of the PA QS-regulon (Wade et al. 2005; Dubern and Diggle 2008; Lee and Zhang 2015). Specifically, we examined signal and receptor gene expression of the three interconnected Las-, Rhl-, and PQS-systems. Finally, we also focused on the regulation of the two siderophores, pyoverdine and pyochelin (Cornelis and Dingemans 2013; Schalk and Cunrath 2016). For one thing, we expect genes involved in the synthesis of siderophores to be differentially expressed between treatments because we explicitly manipulated iron availability. Moreover, it has been shown that siderophores can be deployed as a competitive strategy to suppress the growth of competitors (McKenney et al. 1995; Weaver and Kolter 2004; Joshi et al. 2006; Harrison et al. 2008; Traxler et al. 2013; Tyrrell et al. 2015). This is because specific

receptors are required for the uptake of iron-loaded siderophores, such that secreted siderophores can lock iron away and induce iron starvation for competitors with incompatible receptors. This is the case for pyoverdine produced by PA, for which BC does not possess a receptor (Weaver and Kolter 2004; Leinweber et al. 2017a).

Methods

Bacterial strains and media

For all our experiments, we used the standard laboratory *P. aeruginosa* strain PAO1 (ATCC 15692) and *B. cenocepacia* strain H111 (LMG 23991), an isolate from a cystic fibrosis patient (Gotschlich et al. 2001). Overnight cultures were grown in lysogeny broth (LB). All gene-expression experiments were conducted in casamino acids (CAA) medium (per 1 liter: 5 g casamino acids; 1.18 g $K_2HPO_4 \cdot 3H_2O$; 0.25 g $MgSO_4 \cdot 7H_2O$) supplemented with 20 mM $NaHCO_3$ and 25 mM HEPES buffer. We induced strong iron limitation by adding 100 µg/ml of the natural iron chelator human apo-transferrin. To create iron rich medium, we omitted transferrin from the above recipe and added 20 µM $FeCl_3$ instead. All chemicals for the bacterial culture media were purchased from Sigma-Aldrich, Switzerland.

Supernatant preparation of PA and BC cultures

The goal of our study is to investigate transcriptional changes in PA in response to diffusible and volatile cues from its inter-specific competitor BC (Westhoff et al. 2017). We further intended to find out if the iron availability influences the transcriptional response to competition cues. Therefore, we generated spent culture supernatants from BC and PA monocultures under either low or high iron availability. We argue that secreted substances in spent culture supernatants could contain information on specific environmental conditions and competitors to PA. For instance, the supernatant of BC likely contains secreted enzymes and secondary metabolites (i.e. QS signals, siderophores, hydrolyzing enzymes), which could directly affect or even damage PA cells, thereby informing PA on the presence of a nearby competitor (Loutet and Valvano 2010; Cornforth and Foster 2013; Schwager et al. 2013; Ryan et al. 2015; Westhoff et al. 2017). Similarly, PA could extract information from its own supernatant, also containing secreted enzymes and secondary metabolites (e.g. hydrolyzing enzymes, QS signals, siderophores). These compounds do not harm PA cells due to innate resistance, but might for example affect the QS response or the investment into siderophores.

To generate spent culture supernatants, we first centrifuged LB overnight cultures of PA and BC at 7500 rpm for 2 minutes at room temperature, and washed cell pellets twice with 0.8% NaCl to remove

spent LB medium. We then adjusted the bacteria to an optical density of 1 at 600 nm (OD600) in 0.8% NaCl. Subsequently, we inoculated both iron limited and iron rich CAA medium (150 ml) with 200 µl of the bacterial suspensions (PA or BC) in 500 ml Erlenmeyer flasks, and incubated the cultures at 37°C in an orbital shaker at 220 rpm. We had three biological replicates for each species and iron treatment.

We followed the growth of the PA and BC monocultures by measuring the OD600 regularly in a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences). After reaching stationary phase (after 45.7 h for PA and 48 h for BC in iron limited CAA medium; after 16.8 h for PA and BC in iron rich CAA medium), the cultures were distributed to sterile 50 ml tubes (Sarstedt) and centrifuged at 8000 rpm for 2 minutes at room temperature. We then sterile filtered (PES membrane, 0.22 µm cutoff) the supernatants into new 50 ml sterile tubes and stored them at -80°C until further usage.

Experimental design of transcriptome study

For the main transcriptome experiment, we cultured PA under six different growth conditions in three-fold replication (18 replicates in total). Our main treatment consisted of PA exposed to 30% of BC supernatant mixed with 70% fresh CAA medium. As control treatments, we considered PA growing in 100% fresh medium, and PA being exposed to 30% of its own supernatant mixed with 70% fresh CAA medium. All three treatments were carried out under iron limited and iron rich conditions. Prior to the main experiment, we prepared starter cultures of PA. Specifically, we inoculated two times 20 ml LB medium with 200 µl of a PA overnight culture each in two 100 ml Erlenmeyer flasks for the iron limited and iron rich treatment separately, and incubated these starter cultures at 37°C in an orbital shaker at 220 rpm. Once the cultures reached the late exponential growth phase, we adjusted their OD600 to 1, as described above, and used them to inoculate the 18 experimental cultures (9 each for iron limited and rich treatment) consisting of 200 ml media in one-liter Erlenmeyer flasks, to an initial OD600 of $1x10^{-2}$. For each of the six growth conditions, we cultured a fourth replicate, which we used to monitor the growth trajectories (Figure S1). This was important because growth patterns varied across treatments, and real-time monitoring of culture growth allowed us to harvest all experimental cultures between the mid- and late-exponential phase (Figure S1). Like this, we could rule out that differential gene expression is simply due to variation in nutrient availabilities between treatments.

For harvesting, we transferred the cultures in the Erlenmeyer flasks onto ice. We then distributed them to 50 ml tubes (Greiner) containing 5 ml ice cold stop solution (10% phenol buffered with Tris-HCl to pH8 (Sigma-Aldrich, Switzerland) and 90% ethanol). The suspension was mixed by manual inversion of tubes and centrifuged at 4°C, for 5 minutes at 9000 rpm. The supernatant was discarded, while the cell pellet was flash-frozen in liquid nitrogen and stored at -80°C.

RNA isolation, library preparation and RNA Sequencing

We isolated RNA from the PA cell pellets following the protocol by (Liu et al. 2017). In short, we extracted the total RNA from PA cell pellets using a modified hot acid phenol protocol (Pessi et al. 2007) followed by the removal of short RNAs, including the 5S ribosomal RNA (5S rRNA), with the RNAeasy MiniKit (Qiagen). Genomic DNA was completely removed with RQ1 RNase-Free DNase (Promega), which was followed by purifying the RNA with the RNAeasy MiniKit (Qiagen) and verified by PCR. The quality of the mRNA was confirmed by measuring the rRNA integrity through capillary electrophoresis in a Bioanalyzer 2100 (Agilent) using Agilent RNA 6000 Nano Chips. We used 150 ng of RNA for cDNA synthesis and library preparation with the Encore® Complete Prokaryotic RNA-Seq DR Multiplex System from NuGEN. During the cDNA synthesis, we enriched non-ribosomal RNA due to selective priming. The resulting cDNA was fragmented into 200 bp fragments with a Covaris S220 AFA system prior to the library preparation. We analyzed the concentration, quality and size distribution of the libraries with capillary electrophoresis using D1000 ScreenTape from Agilent (size range 100-800 bp). Single-end sequencing of the libraries was performed on an Illumina HiSeq2500 machine at the Functional Genomics Center Zurich.

Analysis of differential gene expression

Illumina-generated RNA-Seq reads were processed and mapped to the *P. aeruginosa* PAO1 genome sequence (Stover et al. 2000) from the *Pseudomonas* Genome Database (Winsor et al. 2016) using the CLC Genomics Workbench v7.0 (CLC bio). We only considered transcripts that could be unambiguously mapped to non-homologous genes in the PA genome. We further excluded genes with zero or only one unique read from further analysis. We used the Bioconductor package DESeq2 version 1.6.3 (Love et al. 2014) in the R environment version 3.1.2 (R Development Core Team 2014) to analyze differential gene expression. We performed separate analysis for the low and high iron treatments. DESeq2 first determines the size factors of the libraries to correct read counts of single genes, followed by dispersion estimation, which represents the within-group variation. Then a negative binomial general linear model is fit to the data to determine base means (average of normalized counts per gene across all samples), log2 transformed fold changes of gene expression in the treatment relative to the control treatments, and p-values of the test statistics. Wald tests are used to calculate p-values, which are then corrected for multiple testing by the false discovery rate test by Benjamini and Hochberg (Benjamini and Hochberg 1995). For some gene regulations no *p*-value could be assigned. That is the case when one of the replicates constitutes an outlier, which is determined by the Cook's cutoff. In addition, only genes with large enough counts (depending on the basemean and variance of the

expression of the single gene across the respective three replicates) generate sufficient statistical power to yield significant fold changes. We considered fold changes with an adjusted $p < 0.1$ as differentially expressed (Love et al. 2014). To test for clustering of our biological replicates and segregation across treatments we performed a principal component analysis (PCA). For this purpose, we used the regularized logarithm (rlog) transformation (which also normalizes for library size) from the DESeq2 package to increase the similarity of variances of genes with different read counts (Love et al. 2014). For this analysis, we included all expressed genes and analyzed the first three principle components.

Gene set enrichment analysis

In addition to the above analysis, we also conducted a gene set enrichment analysis for our traits of interest, as specified in the introduction. For that purpose, we manually compiled 57 gene sets from the literature covering these traits. We then performed a gene set enrichment analysis (GSEA) using javaGSEA version 2-2.2.4 based on gene lists pre-ranked according to their direction of regulation and their p-values of differential expression in BC relative to PA conditioned medium. The benefit of using GSEA is, that we can analyze general trends in predefined functional groups of genes based on gene expression data that we might not see, when only looking at single genes.

We treated the gene expression data from iron limited and rich conditions as independent data sets. We ensured sufficient statistical power by including only gene sets with at least 5 genes. The normalized enrichment score (NES; normalized for different gene set sizes), which is the primary test statistics of GSEA, shows if genes of one gene set are placed at the top or at the bottom of our preranked gene list, which corresponds to an enrichment in one of the two tested conditions, BC or PA supernatant. We considered gene sets with an FDR q-value < 0.25 (adjusted p-value by false discovery rate test after Benjamini and Hochberg 1995) as significantly enriched (Mootha et al. 2003; Subramanian et al. 2005).

Control experiments and analysis

There are numerous studies that have examined PA gene expression and phenotypic changes in response to iron limitation (Vasil and Ochsner 1999; Blumer and Haas 2000; Bollinger et al. 2001; Lamont et al. 2002; Ochsner et al. 2002; Poole and McKay 2003; Weigert et al. 2017). We used these studies as references to verify whether our transcriptome analyses produced reliable results. First, we conducted a control analysis to test whether our transcriptome data match with those from previous

studies with regard to iron limitation. For this control analysis, we primarily focused on the two siderophores, pyoverdine and pyochelin, which are known to be highly up-regulated under iron limitation, but repressed under iron rich conditions (Dumas et al. 2013). To test whether these patterns can be recovered from our transcriptome analysis, we compared gene regulation of PA growing in iron limited versus iron rich fresh media without supernatants. For pyoverdine and pyochelin, we examined the log2-fold gene expression changes for 17 and 12 genes involved in the regulation, synthesis and uptake of these two molecules, respectively. We further quantified gene expression changes of eight additional genes, whose expressions are known to be directly affected by iron availability, which include: hydrogen cyanide regulation and synthesis, oxidative stress response, and exotoxin A and protease IV, two proteins linked to pyoverdine production (Lamont et al. 2002).

We further conducted a control experiment to examine whether our manipulation of iron availability induced the expected phenotypic changes in pyoverdine production and growth as reported from previous studies (Kümmerli et al. 2009; Dumas et al. 2013). With regard to pyoverdine production, we expect greatly reduced investment in iron rich compared to iron limited media (Dumas et al. 2013). In addition, we expect PA to down-regulate pyoverdine production when growing in its own supernatant compared to fresh media without supernatant. This is because PA can recycle previously produced pyoverdine, which has been shown to curb de-novo production of this molecule (Schalk et al. 2002; Imperi et al. 2009). With regard to growth, we expect cultures to grow significantly better under iron rich compared to iron limited conditions, as iron availability is a key determinant of yield in bacterial cultures. Moreover, we predict that the supplementation of PA supernatant will have opposing effects on PA growth depending on iron availability. Under iron rich conditions, the PA supernatant will mainly consist of spent media, and thus we expect PA growth to be compromised in media supplemented with supernatant compared to fresh media. In contrast, PA supernatant from iron limited conditions contains a lot of the beneficial pyoverdine. Recycling of these molecules was shown to save metabolic costs and boost growth (Imperi et al. 2009; Kümmerli and Brown 2010). Thus, we expect PA to grow better in media supplemented with supernatant as opposed to fresh media.

To test whether we recovered these findings with our transcriptome culturing setup, we grew PA in either fresh CAA medium only or in CAA medium mixed with 30% of PA supernatant, under both iron rich and iron limited conditions, exactly as described above for the main experiment. We adjusted a PA overnight culture to OD600 = 1 and inoculated a 96 well plate (200 µl medium per well) with bacteria to an initial OD600 = $1x10^{-2}$. We then measured the kinetics of growth (OD600) and pyoverdine production (via auto-fluorescence at excitation: 400 nm and emission: 460 nm) in 7 replicates per treatment at 37°C every 15 minutes for 48 hours in a Tecan Infinite M-200 plate reader (Tecan Group Ltd., Switzerland). Prior to each reading event, plates were shaken for 30 seconds (3.5 mm orbital

displacement). To analyze the growth and *de novo* pyoverdine levels we subtracted the blank levels for each treatment separately. This way, we excluded natural turbidity of the medium and the pyoverdine that was already present in the added supernatants from further analysis. We further subtracted the minimum values of OD600 (growth) and fluorescence units (*de novo* pyoverdine production) from each treatment separately to make sure that the starting points of growth and pyoverdine levels for each treatment were equal. We fitted non-parametric spline models to the data, using the grofit R package (Kahm et al. 2010). We used spline fitting because growth and pyoverdine trajectories followed partially non-logistic patterns and varied greatly between the different treatments (Figure S2). For all analyses, we used the integral of the spline models - the area under the curve - as the most robust parameter of growth and pyoverdine production trajectories. We used ANOVA to determine significant differences of the growth and pyoverdine integrals across different media for each iron treatment separately. The false discovery rate test by Benjamini and Hochberg was applied to correct for multiple testing (Benjamini and Hochberg 1995). All statistical analysis was performed under the R environment version 3.1.2 (R Development Core Team 2014).

Results

Burkholderia cenocepacia (BC) supernatant induces global transcriptional changes in Pseudomonas aeruginosa (PA)

We investigated the global gene expression patterns of PA when exposed to the supernatant of its competitor BC in iron rich and iron limited medium, and compared these transcriptomes to those of two control treatments: when PA grows in fresh medium supplemented with its own supernatant (Figure 1a) or in fresh media alone (Figure 1b). Across treatments, we could map between 4.975.289 and 8.592.846 reads from the iron limited conditions, and between 4.213.879 and 7.607.897 from the iron rich treatments to the PAO1 genome. PAO1 has a large genome with over 5500 ORFs (Stover et al. 2000) of which we found 4988 and 4818 genes to be expressed in the iron limited and the iron rich treatment, respectively (Figure 1). When contrasting the transcriptomes of PA exposed to the supernatant of BC versus its own supernatant, we found that 11.4% (in iron limited medium) and 22.2% (in iron rich medium) of all mapped genes were significantly (FDR<0.1) differentially regulated (Figure 1a). We further found that the set of differentially regulated genes differed fundamentally between the iron limited and the iron rich treatment, with only 6.2% of all differentially regulated genes following the same regulation pattern in both environments. Similar patterns emerged when comparing the transcriptomes of PA exposed to the supernatant of BC versus PA growing in fresh medium (Figure 1b).

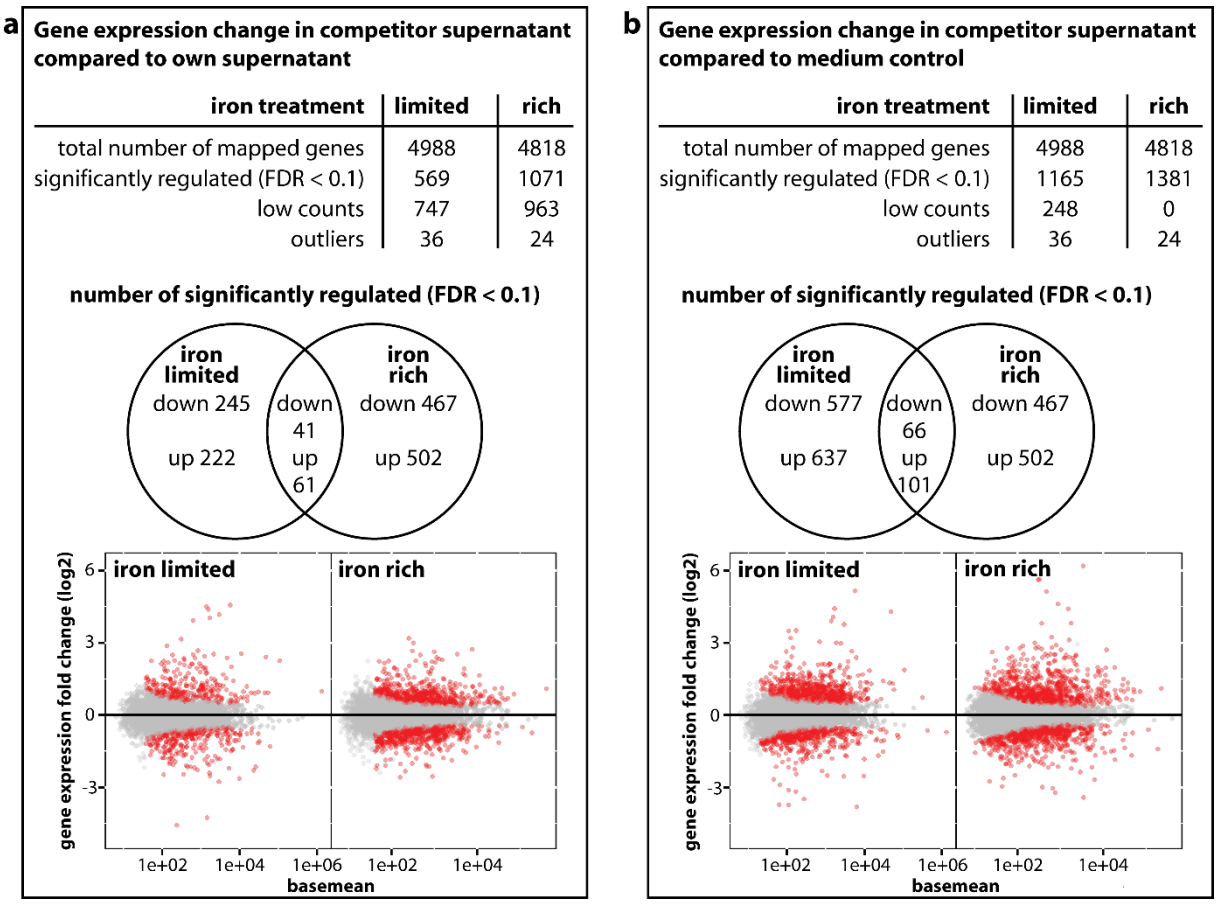


Figure 1: *Summary of our RNA sequencing data of PA grown in a mixture of 70% fresh CAA medium and 30% competitor (BC) supernatant relative to PA grown in either 70% fresh CAA medium mixed with 30% of its own (PA) supernatant* ***(a)*** *or in 100% fresh CAA medium only* ***(b)****. We further investigated if the iron availability modulates the gene expression and therefore grew PA in either iron limited or iron rich conditions. The tables* ***(top)*** *show the number of genes that were expressed and could be unambiguously mapped to the PAO1 genome. Of these genes a certain number were significantly (FDR<0.1) differentially regulated between two treatments. A certain number of the mapped genes had to be excluded from further analysis due to low counts or outliers among the three biological replicates per treatment. The numbers were calculated separately for each comparison of supernatant treatments and for the iron treatment. Venn-diagrams* ***(middle)*** *show the number of genes that were significantly up- or downregulated between two medium treatments in iron limited and rich conditions separately. In the overlapping regions of the circles, the numbers of genes are given that were up- or downregulated together in both iron treatments. The MA-plots* ***(bottom)*** *show the basemean of each gene (mean of gene counts normalized for library size across 6 samples (composed of 3 biological replicates for each of the two respective medium treatments) and its log2 transformed expression fold change between the two respective medium treatments. Each data point constitutes one mapped gene. Red and grey data points show significantly (FDR<0.1) and non-significantly (FDR>0.1) differentially regulated genes, respectively. Note that genes with low basemeans do not yield significant fold changes due to low statistical power.*

To visualize gene expression differences between treatments and check for consistency between replicates of the same treatment, we performed a principal component analysis (PCA) on the rlog transformed and normalized raw count data (Love et al. 2014). We found that the first three principal components (PC) explained 74.9% of the total variance in our data, with the large majority being explained by PC1 (61.43 %), followed by PC2 (6.98 %) and PC3 (6.53%). When contrasting PC1 vs. PC2 (Figure 2a) and PC2 vs. PC3 (Figure 2b), it turns out that PC1 separates iron limited from iron rich

conditions, whereas PC3 separates fresh media from supernatant treatments. Convincingly, we observed that the triplicates of the same treatment always clustered together, demonstrating that our independent biological replicates yielded reproducible results. Because gene expression patterns seemed to be more similar between the two supernatant treatments (i.e. PA with either BC or own supernatant), we decided to focus on this comparison, when analyzing differential gene expression patterns. Although reflecting the more conservative approach, this comparison minimizes the risk of results being influenced by the supernatant vs. fresh media treatment alone.

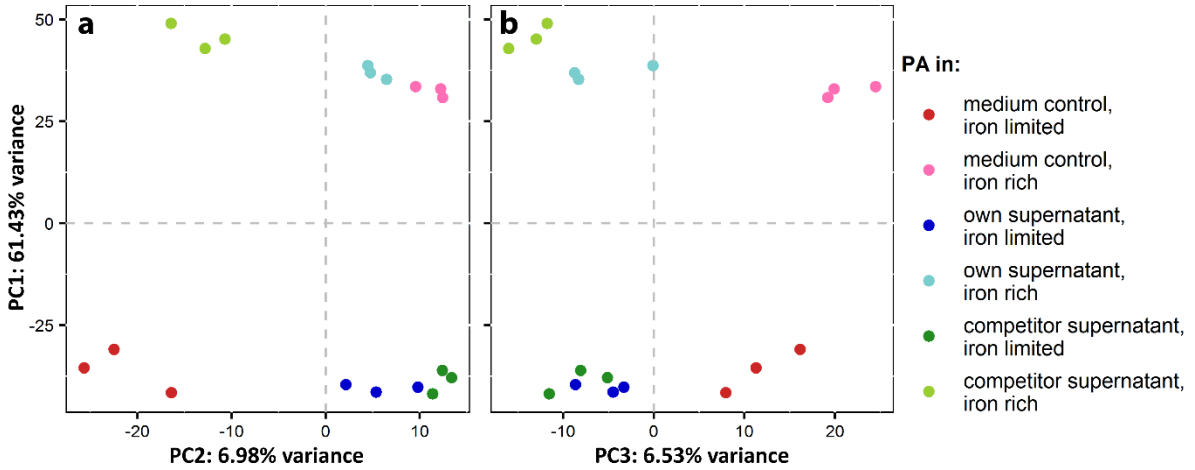


Figure 2: The principal component analysis (PCA) of gene expression of PA grown in different media shows that replicates of one treatment cluster together and that our media treatments (fresh CAA medium with either competitor (BC) supernatant, PA's own supernatant or fresh CAA medium only (medium control)) and the two iron conditions (iron limited or iron rich medium) cause a differential gene expression in PA. We analyzed the first three principal components (PC), which together explain 74.9% of the variance between samples. We compared PC1 vs. PC2 (a) and PC1 vs. PC3 (b). PC1 separates the samples according to the iron treatment and PC3 separates the supernatant treatments from the fresh medium treatments. The variance explained by PC2 cannot be easily explained.

Validation of the RNA-sequencing data and control experiments

There are numerous studies that have examined PA gene expression in response to iron limitation (Vasil and Ochsner 1999; Ochsner et al. 2002; Poole and McKay 2003). Thus, to test whether our RNA-sequencing protocol yields reliable results and recovers the patterns of previous studies, we compared the transcriptome of PA in iron limited versus iron rich medium without the supplementation of any supernatant (Figure 3). Consistent with previous studies, we found that all genes involved in the regulation (pvdS), the synthesis, and uptake (fpvA and fpvB) of the iron-scavenging siderophore pyoverdine were significantly up-regulated under iron limitation (Figure 3). We found the same pattern to hold true for regulatory, synthesis and uptake genes of pyochelin, the secondary siderophore of PA (Stintzi et al. 1999; Lamont and Martin 2003; Poole and McKay 2003). Intriguingly, pyochelin genes

were less dramatically upregulated than pyoverdine genes, exactly matching results from a previous study showing preferential investment into the more efficient pyoverdine under stringent iron limitation (Dumas et al. 2013). Pyoverdine is not only a siderophore, but also a signaling molecule controlling the expression of the virulence factors exotoxinA and the protease IV (Lamont et al. 2002). Consistent with this regulatory link and recent qPCR analysis (Weigert et al. 2017), we found the genes encoding these two virulence factors (*toxA* and *prpL*) to be significantly upregulated under iron limitation (Figure 3).

Besides siderophores, we examined two further traits where iron limitation is known to alter gene expression patterns. The first case considers superoxide dismutases (Sod), which play crucial roles in the oxidative stress response, and depend on metal ions as cofactors. PA expresses two Sod isoenzymes, whereby SodM (=SodA) contains manganese, and SodB iron as cofactor. Bollinger et al. showed that PA switches from the iron dependent SodB to the manganese dependent SodM under iron limitation, exactly the pattern we recovered from out transcriptome data (Figure 3) (Bollinger et al. 2001). The second case involves the synthesis of hydrogen cyanide (HCN), which is positively controlled by the transcriptional regulator ANR. It was shown that the assembly of the Fe-S cluster in ANR is compromised under iron limitation, reducing the expression of HCN synthases encoded by the genes *hcnABC* (Blumer and Haas 2000). We precisely recovered this pattern with our transcriptome data (Figure 3), while the *anr* gene expression itself was not affected by iron limitation. These analyses strongly suggest that our transcriptomics protocol produced reliable results.

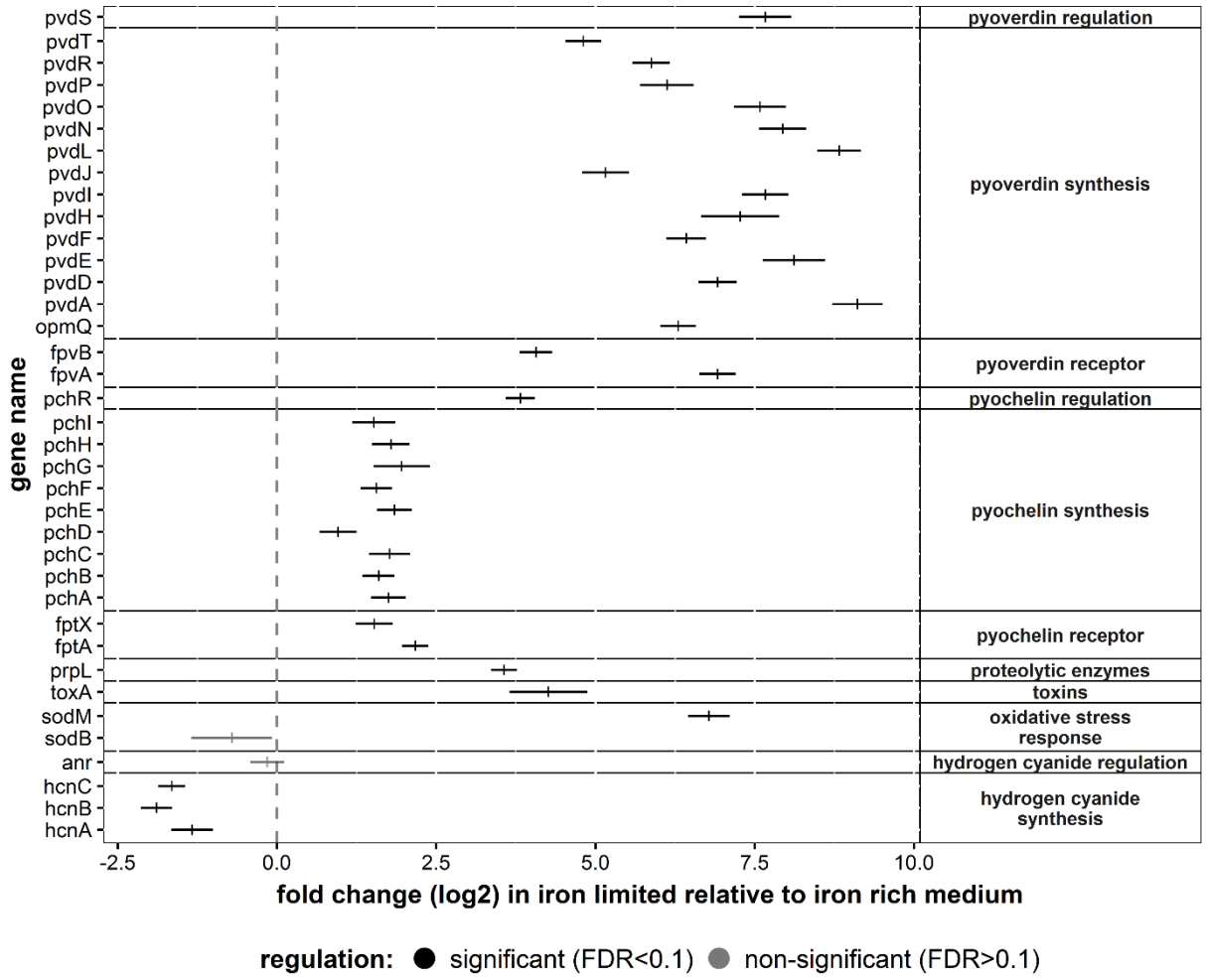


Figure 3: Iron availability of the medium affects PA's gene expression. Genes encoding traits that are known to depend on the iron availability of the environment are also differentially regulated in our RNA sequencing study. Data points left of the dotted line demonstrate a downregulation of gene expression, and right of the dotted line an upregulation of gene expression under iron limitation relative to iron repletion. Vertical and horizontal lines depict the mean ± standard error of the log2 transformed expression fold change of PA genes. We used three biological replicates per treatment. Black and gray lines depict significantly (FDR<0.1) and non-significantly (FDR>0.1) regulated genes, respectively.

Our control experiment tracking pyoverdine production and growth over time recovered the patterns described in earlier studies. Specifically, we observed that PA growth was significantly compromised and pyoverdine production significantly increased in iron limited compared to iron rich medium (Figure 4, Figure S2). Furthermore, and as expected, we found that the supplementation of PA supernatant stimulated the growth of PA under iron limited conditions as it contained recyclable pyoverdine, and thus allowed PA to downscale costly investment into this molecule (Figure 4a+b). Conversely, the supplementation of PA supernatant curbed PA growth under iron rich conditions because it contains little beneficial components, but rather spent media instead (Figure 4c+d).

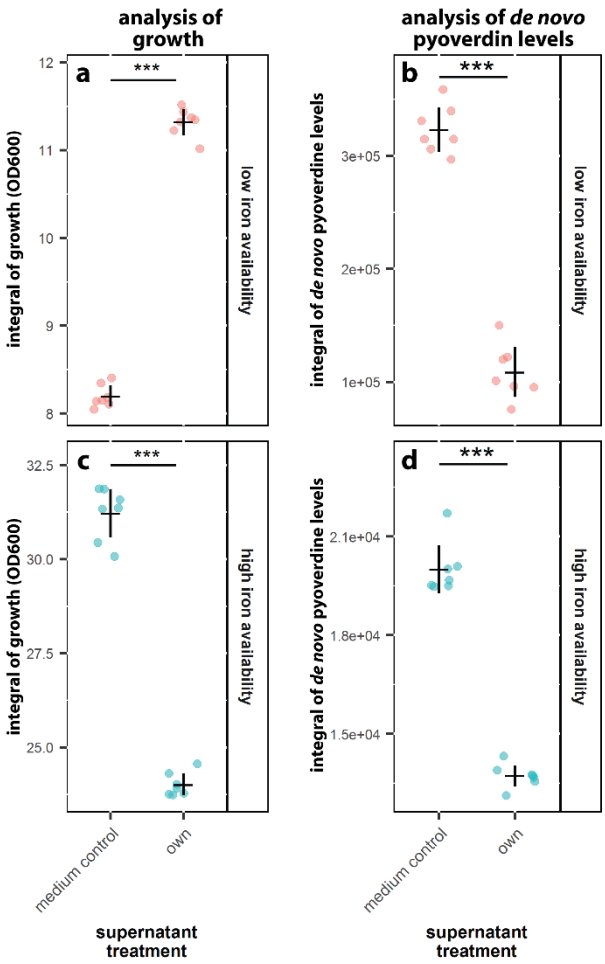


Figure 4: *Growth and de novo pyoverdine production by PA are affected by the iron availability of the medium and the supernatant treatment. Under iron limitation (red), the overall growth of PA is stimulated by its own supernatant due to recyclable pyoverdine in the supernatant (a), and the overall de novo pyoverdine synthesis is reduced (b) compared to growth in CAA medium only (medium control). When iron is replete (blue), PA's own supernatant suppresses growth of PA (c) and de novo pyoverdine synthesis (d) compared to the medium control, because the supernatant mainly consists of spend medium. In general, a higher iron availability leads to higher growth of PA and less de novo pyoverdine synthesis, then when iron availability in the medium is limited. Circles depict individual data points (7 replicates per treatment) and values represent the integral of the growth curve or the integral of the global pyoverdine production profile (area under the curve across 48 hours). Black symbols depict means ± 95% confidence intervals. Asterisks indicate significant (***P<0.0001) differences between PA grown in fresh medium only (medium control) or in fresh medium mixed with its own supernatant.*

Gene set enrichment analysis (GSEA) reveals significant gene expression changes in competitive traits of PA in response to competition cues

We found that the iron availability strongly influences, which pathways are upregulated by PA in response to BC conditioned medium relative to growth in its own supernatant. Under iron limitation, the GSEA showed an enrichment of genes in phenazine synthesis. In contrast, when iron is abundant, genes involved in pyoverdine synthesis, pyocin production and type-6-secretion system (T6SS) were highly expressed in BC conditioned medium relative to medium mixed with PA supernatant (Table 1).

Table 1: Gene set enrichment analysis (GSEA) of traits known to be involved in competition revealed that the iron availability strongly influences which traits are regulated in response to BC diffusible cues. Gene sets with a blue background show upregulated gene sets in PA, when grown in competitor (BC) supernatant (SN) relative to its own (PA) supernatant. Gene sets with a red background show upregulated gene sets in PA, when grown in its own (PA) supernatant relative to the competitor (BC) supernatant. We included only gene sets with >5 genes in the GSEA to ensure sufficient statistical power. Only significant gene sets (FDR q-val <0.25) are shown. The normalized enrichment score (NES; normalized for different numbers of genes in each gene set) is a measure of how strong the entire gene set is upregulated in one of the tested conditions, PA or BC supernatant.

Iron	Upregulation	Enriched gene sets	Number of genes	NES	FDR q-val
limited	BC SN > PA SN	Phenazine synthesis	7	-2.2166	<1E-09
	PA SN > BC SN	Pyochelin synthesis	9	2.1254	0.0001
		Flagella	36	1.7228	0.0292
		Pyocins	26	1.6762	0.0331
		Pyoverdine synthesis	14	1.5588	0.0666
		Proteolytic enzymes	8	1.5604	0.0818
		Type VI secretion system	48	1.4470	0.1095
		Exopolysaccharides	25	1.4266	0.1142
		Type-4a-pili	19	1.2945	0.1987
		T6SS effector and immunity proteins	18	1.3008	0.2067
rich	BC SN > PA SN	Pyoverdine synthesis	13	-2.9463	<1E-09
		Pyocins	23	-2.8179	<1E-09
		Type VI secretion system	48	-1.8881	0.0131
	PA SN > BC SN	Proteolytic enzymes	8	1.6787	0.0322
		Phenazine synthesis	7	1.6188	0.0405

So far, we used GSEA to investigate the regulation of predefined gene sets. However, GSEA is restricted by the size of the gene sets and their functional connection (i.e. encoding functional entities like flagella, or encoding synthesis pathways). However, genes can have multiple functions or are expressed independently from each other (i.e. proteolytic and lipolytic enzymes). Therefore, we will now look further into the regulation of single genes that build the functional groups we used for the GSEA, genes of functional groups with less than 5 genes, and into independently working genes.

The effect of the BC supernatant on the expression of PA siderophore related genes

One strategy in competition with other species could be to upregulate the production of siderophores, in order to bind iron before the competitor can access it (Weaver and Kolter 2004; Harrison et al. 2008; Leinweber et al. 2017b; Niehus et al. 2017; Sexton et al. 2017). In iron rich medium, we indeed found support for this hypothesis. PA upregulated genes involved in the synthesis of its main siderophore pyoverdine when exposed to supernatant of BC (Figure 5). PA further upregulated *fpvA*, the gene encoding the cognate receptor for pyoverdine, whilst down-regulating *fpvB* encoding the receptor typically required for the exploitation of heterologous pyoverdine produced by competing *Pseudomonas* species (Ghysels et al. 2004). Thus, it seems that PA cannot only sense competition by BC, but also recognizes that the competitor is a more distantly related one and not another closely related *Pseudomonas* strain. In iron limited media, in contrast, we observed no pyoverdine gene expression changes in response to BC supernatant. Similarly, we found no evidence for a positive siderophore-related response to competition when considering the expression of genes involved in the synthesis of pyochelin, the secondary siderophore of PA (Figure 5). Under iron limited conditions, we even found pyochelin synthesis and receptor genes (*fptA* and *fptX*) to be down-regulated. Conversely, the pyochelin receptor genes (encoding the outer membrane component FptA, and the inner membrane permease FptX (Michel et al. 2007) were significantly upregulated under iron rich conditions, a pattern that is difficult to interpret. Taken together, our data suggest that the upregulation of siderophores, as a strategy to deprive competitors from iron, does not seem to be universal, but rather applies to specific conditions (here: relatively iron rich media) and certain siderophore types (pyoverdine). However, important to note is that siderophore production is temporarily a highly dynamic process (Kümmerli et al. 2009). For instance, Leinweber et al. found that PA initially up-regulates pyoverdine production in direct competition with BC, but curbs production at later time points during the competition (Leinweber et al. 2017b). This means that measuring mRNA levels at a single time point may not tell us the entire story on how siderophores are deployed against competitors.

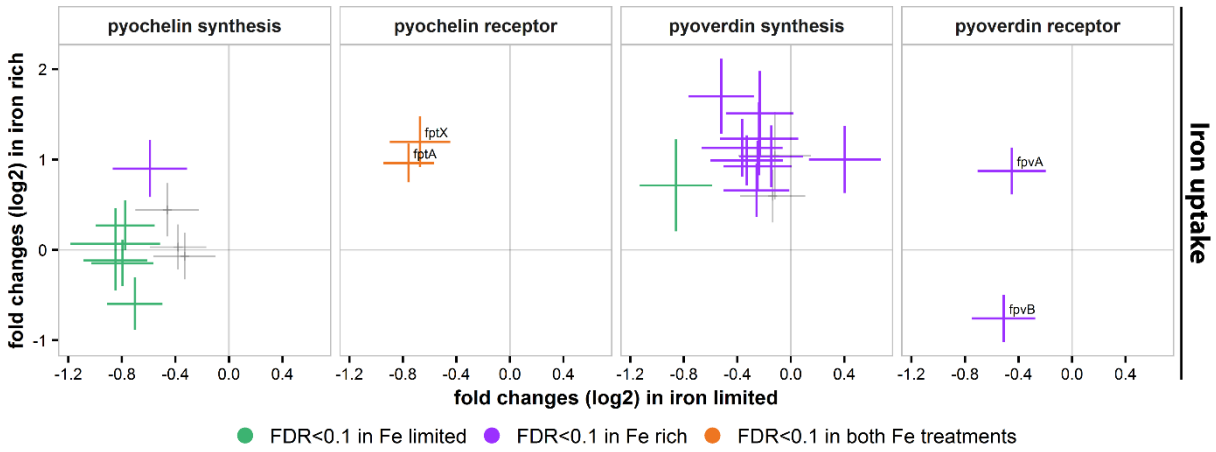


Figure 5: Receiving diffusible cues from the competitor BC leads to changes in the expression of siderophore synthesis and receptor genes in PA. If the genes are up- or downregulated, however, is further modulated by the iron availability of the medium. Gene expression was analyzed in PA grown in BC conditioned medium relative to PA conditioned medium in either iron limited or iron rich conditions separately. Data points depict the mean of the log2 transformed gene expression fold change (log2FC) in PA. We used 3 biological replicates per medium treatment. Vertical and horizontal lines either depict the standard error of the log2FC in iron rich or iron limited medium, respectively. Green, purple and orange symbols represent genes with a significant (FDR<0.1) log2FC in either iron limited, iron rich or in both iron availabilities, respectively. Grey symbols represent genes that are non-significantly (FDR>0.1) regulated due to BC conditioned medium in neither of the two iron treatments.

The BC supernatant stimulates PQS-quorum sensing (QS) related gene expression but not AHL-based QS gene expression

Cell to cell communication in bacterial populations is facilitated by secreted quorum sensing (QS) molecules. They orchestrate the "behavior" of the bacterial population by regulating the expression of many traits in a concentration-dependent manner. These traits can potentially play a role in inter-specific bacterial competition, like biofilm formation or secretion of secondary metabolites and proteins (Schuster and Greenberg 2006; Girard and Bloemberg 2008; Lee and Zhang 2015). It has been shown that environmental factors can influence the synthesis of QS signals and their respective receptors, and thus possibly shift the concentration thresholds for the regulation of target traits (Wagner et al. 2003; Venturi 2006; Williams and Cámara 2009; Mellbye and Schuster 2014; Lee and Zhang 2015). Therefore, we investigated the regulation of QS synthesis and receptor genes of PA in response to BC derived diffusible cues and if the iron availability influences the QS gene expression.

We found, that the expression of PA's two acyl-homoserine lactone (AHL) based autoinducer/transcriptional regulator systems, LasIR and RhlIR, is downregulated when PA receives danger cues through the BC supernatant relative to its own supernatant (Figure 6). LasI and RhlI synthesize the two AHL signaling molecules 3OC12-HSL and C4-HSL produced, which are sensed by the transcriptional regulators LasR and RhlR, respectively (Fuqua et al. 2001). The down regulation of *lasIR* and *rhlIR* happens in iron rich, but not in iron limited medium (Figure 6). PA employs a third QS system,

the *Pseudomonas* quinolone signal (PQS) system. The majority of PQS synthesis genes (*pqsA-E*, *phnAB*) is upregulated in response to BC conditioned medium in both iron treatments, with the two exceptions *pqsH* and *pqsR* (Figure 6). PqsH catalyzes the transformation of the precursor HHQ to the final PQS molecule, which would then bind to its cognate receptor and transcriptional regulator PqsR (Wade et al. 2005; Dubern and Diggle 2008).

The three QS systems of PA are strongly interlinked, with LasIR being the top regulator. The LasIR system positively regulates the PQS systems, LasIR and PQS together stimulate the RhlIR system, and the Rhl signal inhibits the PQS system in a negative feedback loop (Wade et al. 2005; Dubern and Diggle 2008; Lee and Zhang 2015). Our findings could be explained by the following scenario, which is reviewed by Dubern and Diggle 2008 When LasIR is less expressed, *rhlIR*, *pqsR* and *pqsH* lack the positive stimulus. This leads to an increased expression of *pqsA-E* and *phnAB*, due to the lack of inhibition by RhlIR.

The downregulation of the *pqsH* expression could indicate an accumulation of the PQS precursor HHQ. This molecule can function as a QS signal via the PqsR receptor, but it is a weaker transcriptional regulator then PQS and cannot stimulate the expression of phenazines, which depend on PQS itself (Diggle et al. 2007). Furthermore, HHQ has a bacteriostatic effect on many gram-negative bacteria (Reen et al. 2011). Although this effect was not tested for BC, it is possible that the gram-negative BC could be negatively affected by HHQ. Thus, additionally to its role as QS molecule, HHQ could also function as a means for direct interference competition between PA and BC.

In summary, competition cues from the BC supernatant negatively affect QS expression in PA, but potentially leads to the accumulation of the bacteriostatic secondary metabolite HHQ.

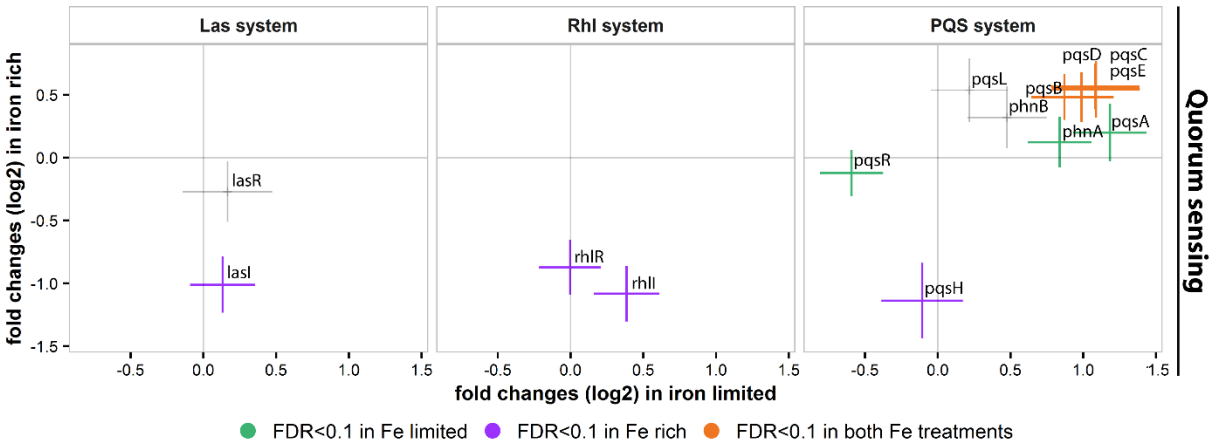


Figure 6: PA's quorum sensing (QS) synthesis and receptor genes are differentially regulated in response to diffusible cues from the competitor BC. The genes encoding the synthesis of HHQ, a PQS precursor molecule, constitute the only tested gene set, that is upregulated in high and low iron availability alike. Gene expression was analyzed in PA grown in BC conditioned medium relative to PA conditioned medium in either iron limited or iron rich conditions separately. Data points depict the mean of the log2 transformed gene expression fold change (log2FC) in PA. We used 3 biological replicates per medium treatment. Vertical and horizontal lines either depict the standard error of the log2FC in iron rich or iron limited medium, respectively. Green, purple and orange symbols represent genes with a significant (FDR<0.1) log2FC in either iron limited, iron rich or in both iron availabilities, respectively. Grey symbols represent genes that are non-significantly (FDR>0.1) regulated due to BC conditioned medium in neither of the two iron treatments.

The BC supernatant affects motility related genes in PA but has little effect on the expression of PA genes encoding adhesion and biofilm formation

Biofilm formation and increased motility can be used to avoid competition in polymicrobial communities by either protecting bacteria from biotic stress like bactericidal substances or by moving away from competitors (Mah and O'Toole 2001; Hall-Stoodley et al. 2004; Hoffman et al. 2005; Kaiser 2007; Marr et al. 2007; Kaplan et al. 2012; Graff et al. 2013; Vega and Gore 2014, 2015; Oliveira et al. 2015). In our analysis of PA gene expression in response to diffusible cues from the competing species BC, the genes responsible for biofilm formation where either not affected or in the case of rhamnolipd synthesis genes downregulated under high iron availability. In contrast, our analysis showed, that PA increases the expression of structural flagella genes upon sensing BC derived cues under high iron availability, while these genes are generally downregulated in iron limited BC conditioned medium (Figure 7, Table 1). These results can be interpreted in two different ways. First, flagella motility is usually a directed movement along chemical gradients, governed by chemotaxis. The direction of movement depends on the attracting or repellent properties of the compounds (Sourjik 2004; Sampedro et al. 2015). Thus, secreted cues from BC could have a repellent effect on PA leading to initiation of an avoidance strategy in PA. Second, a higher motility could lead to a fitness advantage of the highly motile bacterium in competition by increasing access to nutrients (Sampedro et al. 2015). For example PA uses flagella to move towards higher concentrations of oxygen at the air-liquid-

interface, where it can form pellicles (Yamamoto et al. 2012; Hölscher et al. 2015). Pellicles are free-floating biofilms and the role of flagella in their formation illustrates the general role of flagella for biofilm formation and dispersal in PA. The common model of biofilm formation in PA is a multi-step process. PA uses its one polar flagellum to swim towards a biotic or abiotic surface, where PA moves across the surface in a twitching motion with type 4a pili (Tomich et al. 2007; Burrows 2012), to find a suitable position. There, PA firmly attaches to the surface and forms microcolonies, which develop into mature biofilms (Sauer et al. 2002; Stoodley et al. 2002). The extracellular matrix of biofilms consists of cooperatively secreted extracellular DNA, rhamnolipids, and the exopolysaccharides pel, psl and alginate (Ryder et al. 2007). Attachment of PA to the extracellular matrix and biotic or abiotic surfaces is mediated by cup-fimbriae (Giraud and de Bentzmann 2012),the adhesin CdrA (Borlee et al. 2010), lectins PA-IL and PA-IIL encoded by the genes *lecA* and *lecB* (Imberty et al. 2004), and type 4 tight adherence (tad) pili (Tomich et al. 2007; Burrows 2012). Finally, PA can disperse from the biofilm structure via swimming movement, which is mediated by PA's flagella. Dispersion can be induced by nutrient depletion or a hostile environment (Kim and Lee 2016). Thus, PA's swimming motility and biofilm formation are functionally connected. The environment has a strong effect on both phenotypes, and the biofilm formation is further regulated by QS (Winzer et al. 2000; Fazli et al. 2014; Sampedro et al. 2014). Therefore, PA could switch between a planktonic and sessile lifestyle on different time points during growth.

Although biofilm formation is generally considered a mechanism to protect bacteria from diffusible toxic compounds (Vega and Gore 2014, 2015; Oliveira et al. 2015), we found in our PA gene expression study that PA increases the expression of flagella assembly genes, which could indicate that PA either avoids distant competitors by moving away from them or that it moves towards higher nutrient concentrations to gain a fitness advantage over its competitors. Nevertheless, since biofilm formation and swimming motility are highly complex processes as described above, it is possible that PA induces the biofilm formation during later growth in response to diffusible danger cues from BC in our experiments.

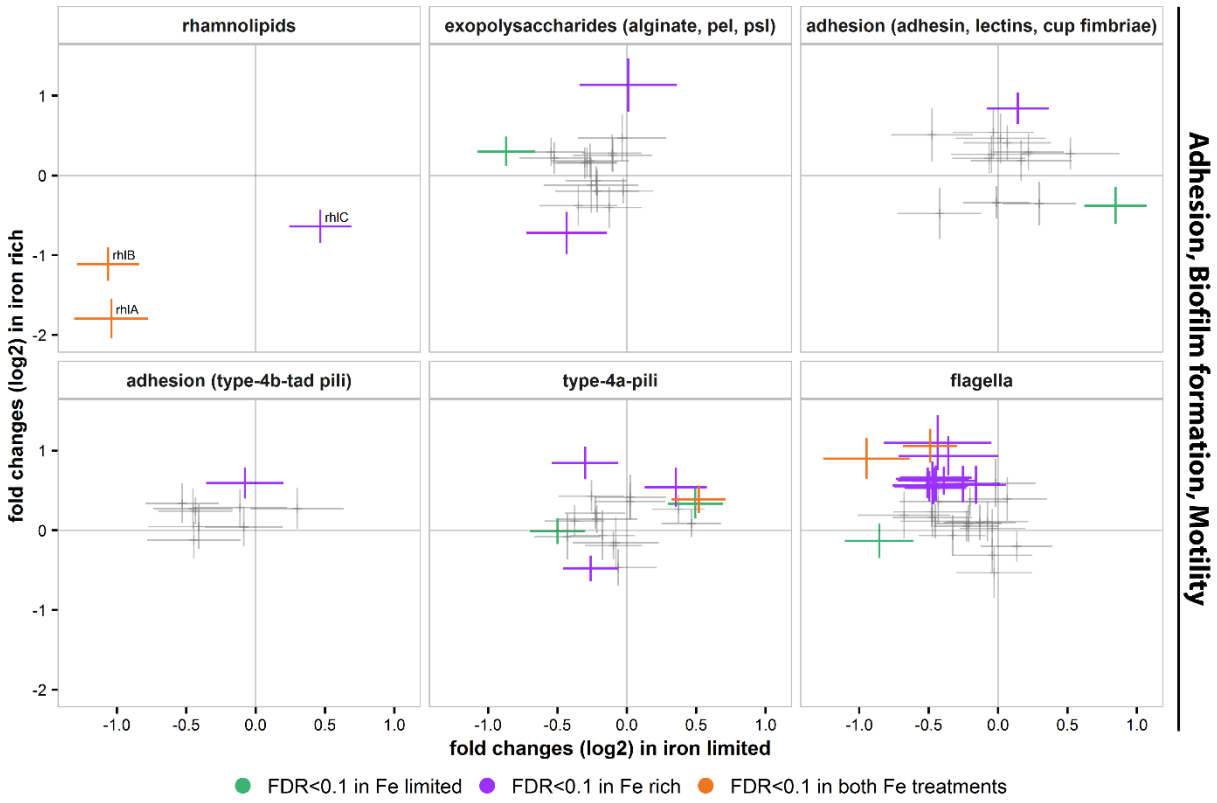


Figure 7: Genes that play a role in the biofilm formation of PA (see text for information) are generally not or negatively affected by BC derived diffusible danger cues. In contrast, PA invests in higher motility under iron repletion by increasing the gene expression of flagella assembly genes. Gene expression was analyzed in PA grown in BC conditioned medium relative to PA conditioned medium in either iron limited or iron rich conditions separately. Data points depict the mean of the log2 transformed gene expression fold change (log2FC) in PA. We used 3 biological replicates per medium treatment. Vertical and horizontal lines either depict the standard error of the log2FC in iron rich or iron limited medium, respectively. Green, purple and orange symbols represent genes with a significant (FDR<0.1) log2FC in either iron limited, iron rich or in both iron availabilities, respectively. Grey symbols represent genes that are non-significantly (FDR>0.1) regulated due to BC conditioned medium in neither of the two iron treatments.

PA selectively increases expression of type-6-secretion system (T6SS) and T5SS genes when receiving secreted cues from BC under iron repletion

PA features a vast diversity of protein secretion systems. Up to now, there are five different secretion systems known in PA: type-1-secretion-system (T1SS), T2SS, T3SS, T5SS and T6SS, which can be further categorized into sub-systems. Due to their diverse structures and mechanisms, the different secretion systems can fulfill various functions. They have been found to play crucial roles in colonizing new habitats, scavenging nutrients, establishing infections and competing against other bacteria. The diverse protein secretion systems of PA can precisely deliver their effector proteins to locations where they can fulfil their functions. They can precisely target their effector proteins either into the extracellular environment (T1SS, T2SS, T5SS) or inject effector proteins directly into target cells (T3SS, T6SS) (Bleves et al. 2010; Filloux 2011). Therefore, protein secretion systems are important means for

PA to influence its environment and could be differentially expressed in response to secreted cues from other bacterial species in order to mediate inter-specific competition.

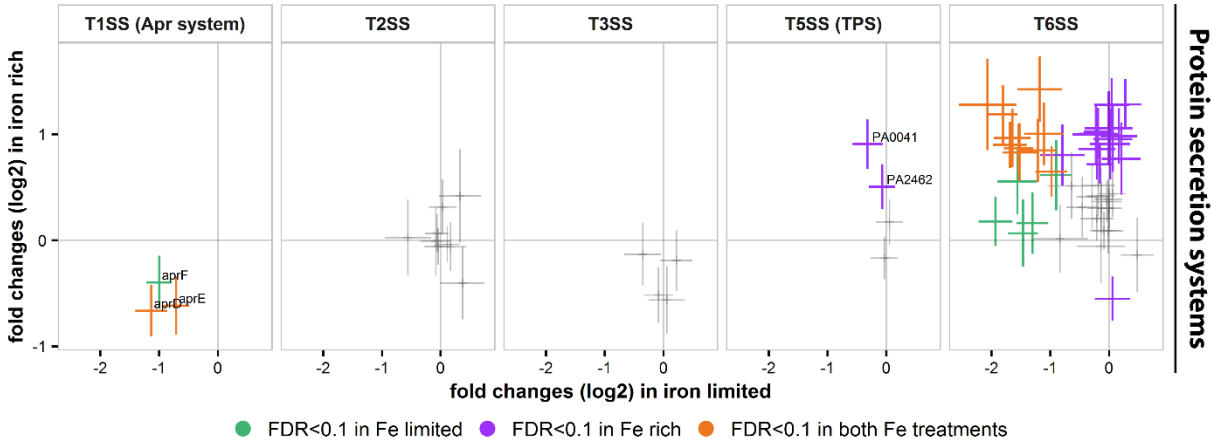


Figure 8: When RA receives diffusible cues from BC in iron replete medium, it upregulates genes encoding contact dependent inhibition mechanisms, the type-6-secretion system (T6SS) and two of the T5SS. The transcription of PA genes coding for the general secretion of proteins into the medium (by T1SS and T2SS) and the injection mechanism of proteins into eukaryotic cells (T3SS) is either negatively affected or not regulated in BC conditioned medium. Gene expression was analyzed in PA grown in BC conditioned medium relative to PA conditioned medium in either iron limited or iron rich conditions separately. Data points depict the mean of the log2 transformed gene expression fold change (log2FC) in PA. We used 3 biological replicates per medium treatment. Vertical and horizontal lines either depict the standard error of the log2FC in iron rich or iron limited medium, respectively. Green, purple and orange symbols represent genes with a significant (FDR<0.1) log2FC in either iron limited, iron rich or in both iron availabilities, respectively. Grey symbols represent genes that are non-significantly (FDR>0.1) regulated due to BC conditioned medium in neither of the two iron treatments.

Our results show, that the transcription of genes encoding protein secretion systems that do not inject their protein load into target cells but release enzymes into the medium, are either significantly downregulated in the case of T1SS, or not significantly regulated in the case of T2SS upon receiving secreted cues from the competitor BC. The iron availability of the medium does not seem to further influence this gene expression (Figure 8).

The T1SS is structurally one of the simplest secretion systems in bacteria. It consists of an outer membrane protein, an ATP-binding cassette (ABC) transporter in the inner membrane and a connecting protein. PA expresses two T1SS. First, the Apr-T1SS, which secrets an alkaline protease AprA and one protein of still unknown function called AprX. Second, the Has system, which is crucial for heme uptake by PA, and thus plays only a role in the pathogen-host context (Bleves et al. 2010; Filloux 2011). Since our medium does not contain heme that could be used as an iron source by PA, we excluded the genes encoding the Has-system from our transcriptional study (Cornelis 2010).

PA carries two different T2SS. The Xcp-T2SS is responsible for secreting most extracellular lipolytic and proteolytic enzymes into the environment that can degrade macromolecules as a source of nutrients for PA. These hydrolyzing enzymes are further important virulence factors contributing to establish

stable infections of PA inside host organisms. Hence, the Xcp-T2SS plays an important role in nutrient uptake, virulence, and potentially in inter-specific bacterial competition (Cianciotto and White 2017). The second T2SS is called the Hxc-T2SS, and is so far only known to be expressed in phosphate-limited conditions. It secretes only one enzyme, the phosphatase LapA (Ball et al. 2002). In our study, we detected none or only very low copy numbers of the *Hxc* gene transcripts, indicating that the media in our experiments were not phosphate limited (Figure 8).

The transcription of T5SS encoding genes in response to BC derived diffusible cues is more diverse and further modulated by the iron availability. The group of T5SS is build-up of separate secretion systems, which are specific for only one passenger enzyme each and function independently from each other. One specific subgroup of T5SS are the two-partner-secretion (TPS) systems that consist of one secreted enzyme, the passenger protein, which is transported across the outer membrane by one specific transporter protein. These transporters are large β-barrel proteins in the outer cell membrane (Bleves et al. 2010; Filloux 2011). We found the genes for the T5SS transporter proteins to be expressed at very low levels or to be non-significantly regulated in BC conditioned medium, independent of the iron conditions. The genes encoding the passenger proteins however, are differentially regulated, although transporter and passenger genes are mostly organized in operons. The expression of genes coding for the passenger proteins PA0041 and PA2462 is significantly upregulated by PA in the BC conditioned medium when iron is abundant (Figure 8). The different amounts of transcripts of the transporter and passenger genes of TPS-systems indicate that other regulatory mechanisms on the post-transcriptional level might be in place. Furthermore, it is possible that, once the transporter protein is assembled in the outer membrane, it could secrete several passenger proteins, which could explain why we detected higher amounts of passenger protein mRNA in our experiments. The passenger proteins of TPS-systems fulfill different functions in PA (Bleves et al. 2010; Filloux 2011). The secreted protein LepA is an extracellular protease, CdrA functions as an adhesin binding the psl polysaccharide of the extracellular matrix of biofilms. Other TPS secreted enzymes are important virulence factors under low phosphate conditions (PdtA), or have been shown to possibly mediate contact dependent inhibition (PA0041 and PA2463) (Borlee et al. 2010; Filloux 2011; Faure et al. 2014; Mercy et al. 2016). The fact that PA increases the expression of the genes PA0041 and PA2463 in response to BC derived secreted cues could indicate that PA invests in contact dependent inhibition mechanisms before having cell-cell contact with an inter-specific competitor (Mercy et al. 2016).

Of the protein secretion systems that directly inject effector proteins into target cells, we only found the T6SS to be significantly regulated by PA upon growth in BC conditioned medium. However, the direction of gene the gene expression (up/down-regulation) depended on the iron availability. The expression of T3SS genes did not significantly change in our experiments (Figure 8, Table 1).

The T3SS of PA directly injects its effector proteins into eukaryotic target cells, which is why the T3SS is one of the most important PA virulence factors, contributing to the degradation of host tissue and manipulation of immune system (Hayes et al. 2010).

The T6SS are very common among gram-negative bacteria, and many of them feature more than one T6SS, so that the bacteria can expand their target spectrum or regulate the separate T6SS according to different environmental cues. PA expresses three T6SS, with the H1-T6SS being specific for only prokaryotic target cells, whereas the H2- and H3-T6SS can inject effectors into prokaryotic and eukaryotic cells. Each of the three systems injects unique effectors proteins into its target cells (Russell et al. 2011; LeRoux et al. 2012; Li et al. 2012; Chen et al. 2015; Sana et al. 2016). In our experiments the H1- and H2-T6SS gene expression is significantly upregulated in iron rich medium, whereas it is downregulated under iron limitation, upon growth in BC relative to growth in PA conditioned medium. Genes coding for structural proteins, that build the H3-T6SS are mostly non-significantly regulated (Table 2). The T6SS functions like a syringe consisting of a contractible sheath that catapults a needle with a puncturing device at the tip through its outer membrane into a target cell and injects effector proteins. The T6SS machinery spans from the cytosol through both bacteria membranes to the outside of the cell. The injection process is ATP-dependent, which is provided by a cognate ATPase (Silverman et al. 2012; Chen et al. 2015). An attack by the T6SS, can be triggered by another T6SS attack in a tit-for-tat kind of way (Basler et al. 2013) suggesting that cell-cell contact is important for the functioning of the T6SS (Hayes et al. 2010; Konovalova and Søgaard-Andersen 2011; Basler and Mekalanos 2012). There are also indications that the H1-T6SS is expressed upon sensing diffusible danger signals like lysed kin cells and sub-inhibitory concentrations of antibiotics (Jones et al. 2013; LeRoux et al. 2015a; Westhoff et al. 2017). The H2-T6SS can be upregulated in iron limitation in a Fur dependent manner (Sana et al. 2012) and H2- and H3-T6SS have been shown to be maximally activated during stationary phase indicating a regulation via QS at high cell densities (Sana et al. 2012, 2013). Nevertheless, T6SS needs physical contact with a target cell to carry out an attack. Our results indicate that diffusible danger cues derived from the competitor BC can also mediate the expression of T6SS in PA. Our results further demonstrate that these cues lead to a differential regulation of H1-, H2 and H3-T6SS structural genes. The regulation of T6SS encoding genes is further modulated by the iron availability. Thus, the danger cues from BC could be interpreted as an announcement of an upcoming threat, upon which PA invests in the assembly of contact-dependent-inhibition mechanism, as we discussed above for the T5SS, to be prepared for future direct cell-to-cell competition.

***Table 2**: Summary of gene expression analysis of PA genes that encode the H1-, H2- and H3-T6SS and respective effector and immunity proteins. The gene expression was analyzed in PA grown in BC conditioned medium relative to PA conditioned medium in either iron limited or iron rich conditions separately. The table summarizes the Base Mean (number of unique reads corrected for library size across all samples of the two medium treatments), the log2 transformed gene expression fold change (log2FC), the standard error (SE) of the log2FC, and FDR adjusted p-value (padj) of the log2FC. The table contains only genes, of which transcripts could be detected in at least one of the two iron treatments. Entries with purple, green or orange background show genes that are significantly (padj ≤ 0.1) differentially regulated in iron rich, iron limited or in both iron conditions, respectively. Entries written in grey depict genes, whose p-value could not be assigned in one of the two or both iron conditions.*

				Iron limited conditions				Iron rich conditions			
system	**function**	**Locus Tag**	**Gene name**	**Base Mean**	**log2FC**	**SE**	**padj**	**Base Mean**	**log2FC**	**SE**	**padj**
H1-T6SS	structural	PA0090	clpV1	383.912	-0.458	0.266	0.334	800.383	0.312	0.289	0.483
		PA0085	hcp1	172.736	-0.192	0.319	0.845	556.885	1.005	0.236	0.000
		PA0086	hsiE1	100.819	0.019	0.338	0.994	111.774	0.908	0.330	0.032
		PA0077	icmF1	330.362	-0.189	0.242	0.777	895.647	1.027	0.224	0.000
		PA0082	tssA1	141.304	0.482	0.281	0.336	75.786	-0.138	0.352	0.829
		PA0083	tssB1	1945.925	-0.053	0.289	0.971	2530.669	1.357	0.246	NA
		PA0084	tssC1	464.872	0.280	0.271	0.665	763.477	1.283	0.236	0.000
		PA0087	tssE1	33.953	-0.051	0.441	NA	31.061	1.346	0.466	NA
		PA0088	tssF1	282.272	-0.206	0.317	0.832	444.317	0.862	0.291	0.019
		PA0089	tssG1	136.792	0.063	0.334	0.970	305.404	0.954	0.309	0.014
		PA0080	tssJ1	27.312	-0.225	0.394	NA	90.472	0.999	0.282	0.004
		PA0079	tssK1	121.198	0.046	0.302	0.978	277.204	1.279	0.249	0.000
		PA0078	tssL1	84.903	0.167	0.311	0.871	246.174	0.983	0.262	0.002
		PA0091	vgrG1	66.529	-0.643	0.404	0.392	163.643	0.514	0.313	0.245
		PA2685	vgrG4	298.195	-0.134	0.232	0.855	192.824	0.305	0.238	0.387
	secreted enzyme	PA2702	tse2	510.560	0.337	0.269	0.550	300.092	0.123	0.274	0.803
		PA3484	tse3	219.329	-0.190	0.283	0.824	145.570	0.761	0.340	0.094
		PA2774	tse4	17.874	0.400	0.484	NA	NA	NA	NA	NA
		PA2684	tse5	361.527	-0.153	0.228	0.824	526.143	1.134	0.213	0.000
		PA0093	tse6	219.471	-0.171	0.261	0.830	265.256	0.485	0.318	0.285
		PA0099	tse7	468.587	-0.359	0.288	0.554	1218.338	1.162	0.249	NA
	immunity protein	PA1845	tsi1	25.057	0.940	0.478	NA	NA	NA	NA	NA
		PA2703	tsi2	19.929	0.248	0.441	NA	NA	NA	NA	NA
		PA3485	tsi3	39.839	-0.240	0.428	0.863	21.855	0.429	NA	NA
		PA2775	tsi4	14.699	0.022	0.472	NA	NA	NA	NA	NA
		PA0092	tsi6	22.524	0.623	0.445	NA	24.657	-0.480	0.452	NA
H2-T6SS	structural	PA1662	clpV2	189.769	-0.981	0.275	0.005	332.704	0.648	0.241	0.037
		PA1668	dotU2	217.019	-1.934	0.286	0.000	299.465	0.178	0.233	0.643
		PA5267	*hcpB*	*192.613*	*-2.070*	0.489	*0.001*	*810.208*	*1.281*	0.432	*0.019*
		PA1656	hsiA2	303.756	-1.305	0.274	0.000	277.247	0.162	0.290	0.747

				Iron limited conditions				Iron rich conditions			
system	**function**	**Locus Tag**	**Gene name**	**Base Mean**	**log2FC**	**SE**	**padj**	**Base Mean**	**log2FC**	**SE**	**padj**
		PA1657	*hsiB2*	*383.239*	*-1.646*	0.313	*0.000*	*772.213*	*0.966*	0.276	*0.004*
		PA1658	*hsiC2*	*2738.799*	*-1.807*	0.252	*0.000*	*7789.338*	*1.189*	0.275	*0.000*
		PA1659	hsiF2	67.163	-0.794	0.377	0.197	90.159	0.803	0.287	0.028
		PA1660	hsiG2	127.400	-1.109	0.338	0.013	131.103	1.004	0.294	0.006
		PA1661	hsiH2	73.175	-1.182	0.380	0.022	78.947	1.424	0.310	0.000
		PA1667	hsiJ2	343.029	-1.687	0.285	0.000	688.097	0.901	0.220	0.001
		PA1669	icmF2	731.169	-1.539	0.254	0.000	1758.639	0.869	0.231	0.002
		PA1666	lip2	114.324	-1.526	0.287	0.000	254.804	0.830	0.275	0.017
		PA1663	sfa2	119.596	-1.562	0.339	0.000	181.022	0.556	0.309	0.198
		PA1671	stk1	70.276	-1.214	0.317	0.002	125.512	0.848	0.298	0.025
		PA1511	vgrG2a	53.553	-0.141	0.409	0.932	47.959	-0.055	0.352	0.931
		PA0262	vgrG2b	55.039	-0.004	0.406	0.998	63.057	1.056	0.350	0.017
	secreted enzyme	PA3487	pldA	641.053	-1.358	0.243	0.000	764.090	0.855	0.237	0.003
		PA3290	tle1	215.019	-0.537	0.282	0.269	248.243	0.505	0.247	0.132
		PA0260	tle3	444.502	-0.076	0.214	0.929	488.336	0.507	0.189	0.037
		PA1510	tle4	90.405	-0.176	0.316	0.864	68.508	0.570	0.324	0.210
	immunity protein	PA3291	tli1	NA	NA	NA	NA	13.634	-0.187	0.491	NA
		PA0259	tli3	888.169	-0.372	0.216	0.336	891.440	0.475	0.202	0.076
		PA1509	tli4	1058.000	-0.830	0.207	0.001	2443.672	0.206	0.217	0.551
		PA3488	tli5a	294.490	-1.222	0.243	0.000	508.501	0.509	0.237	0.110
H3-T6SS	structural	PA2371	clpV3	2071.164	-0.083	0.242	0.933	1501.171	0.094	0.176	0.761
		PA2362	dotU3	53.857	0.210	0.333	0.838	85.106	0.769	0.340	0.089
		PA2367	hcp3	715.288	0.057	0.236	0.959	683.937	0.440	0.221	0.144
		PA2360	hsiA3	312.299	-0.018	0.264	0.993	453.251	0.088	0.213	0.818
		PA2365	hsiB3	12217.320	-0.156	0.225	0.815	3914.774	0.718	0.182	0.001
		PA2366	hsiC3	11611.724	0.001	0.215	0.998	8107.664	0.303	0.191	0.261
		PA2368	hsiF3	252.159	-0.288	0.266	0.640	157.648	0.412	0.311	0.368
		PA2369	hsiG3	550.096	-0.140	0.240	0.852	421.305	0.422	0.252	0.235
		PA2370	hsiH3	480.683	-0.184	0.285	0.832	282.635	0.516	0.290	0.204
		PA2363	hsiJ3	236.248	-0.209	0.283	0.798	280.058	0.205	0.211	0.539
		PA2361	icmF3	848.870	-0.041	0.256	0.977	927.267	0.364	0.191	0.166
		PA2364	lip3	162.366	-0.069	0.388	0.972	NA	NA	NA	NA
		PA2359	sfa3	56.493	-0.228	0.387	0.851	39.189	0.769	0.366	NA
		PA2373	vgrG3	1564.158	-0.012	0.238	0.995	1953.515	0.388	0.189	0.130
	secreted enzyme	PA5089	pldB	88.554	0.011	0.313	0.996	206.820	-0.211	0.298	0.671

				Iron limited conditions				Iron rich conditions			
system	**function**	**Locus Tag**	**Gene name**	**Base Mean**	**log2FC**	**SE**	**padj**	**Base Mean**	**log2FC**	**SE**	**padj**
	immunity protein	PA5086	tli5b1	124.353	0.316	0.336	0.705	532.943	0.181	0.257	0.673
		PA5087	tli5b2	39.309	-0.096	0.399	0.960	99.547	0.089	0.316	0.879
		PA5088	tli5b3	130.741	0.116	0.299	0.920	300.982	-0.382	0.249	0.282

BC derived diffusible cues cause transcriptional changes of PA genes coding for the synthesis of secreted toxic secondary metabolites and for secreted enzymes

PA is a ubiquitious bacterium that lives in habitats as different as marine ecosystems and human hosts in multispecies communities where copetition for common resources and space is high (Aujoulat et al. 2012). To persist under these circumstances, PA expresses an armory of molecular weapons like secondary metabolites and enzymes to scavenge nutrients, to defend itself and to outcompete bacterial competitors (Bleves et al. 2010; Hibbing et al. 2010; Stubbendieck and Straight 2016). We found in our experiments several of these mechanisms to be differentially regulated in response to diffusible cues derived from PA's competitor BC. The direction of the gene regulation depended on the iron availability of the medium with rare overlaps. In particular, we found the genes for pyocin synthesis, H1- and H2-T6SS effectors, and the genes encoding the protease ImpA and the lipoxygenase LoxA upregulated upon BC competition cues in iron rich medium. Under iron limited conditions the BC conditioned medium causes an increased transcription of the hydrogen cyanide and phenazine synthesis genes, and the gene for PA's main lipase LipA. The expression of the genes coding for the phospholipase C PlcB and the aminopeptidase PaaP is downregulated by PA, when grown in BC supernatant under both iron availabilities (Figure 9, Table 2+3).

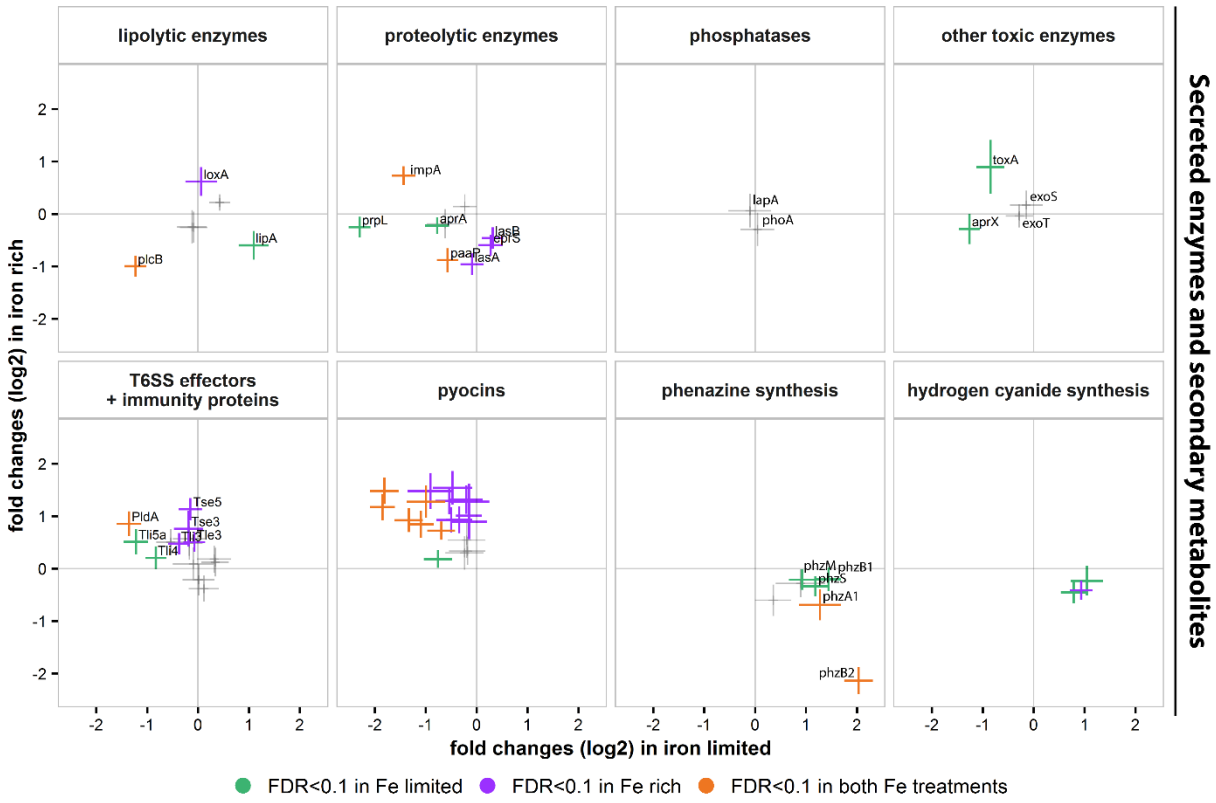


Figure 9: Diffusible cues from PA's competitor BC induce a strong transcriptional response in genes coding for secreted enzymes and toxic secreted metabolites. The gene regulation of specific traits is further strongly modulated by the iron availability of the medium. Gene expression was analyzed in PA grown in BC conditioned medium relative to PA conditioned medium in either iron limited or iron rich conditions separately. Data points depict the mean of the log2 transformed gene expression fold change (log2FC) in PA. We used 3 biological replicates per medium treatment. Vertical and horizontal lines either depict the standard error of the log2FC in iron rich or iron limited medium, respectively. Green, purple and orange symbols represent genes with a significant (FDR<0.1) log2FC in either iron limited, iron rich or in both iron availabilities, respectively. Grey symbols represent genes that are non-significantly (FDR>0.1) regulated due to BC conditioned medium in neither of the two iron treatments.

We found that danger cues from the BC supernatant lead to a significantly increased transcription of genes involved in phenazine synthesis. Transcription of *phzM* and *phzS* was upregulated under iron limitation, but not under iron repletion. *PhzH* is not differentially regulated due to the tested conditions (Figure 9, Table 1). PA expresses a number of phenazines with anti-microbial properties (Sorensen and Klinger 1987; Mavrodi et al. 2001). The precursor of the phenazines, phenazine-1-carboxylic acid (PCA), is synthesized by the products of two homologous gene clusters *phzA1-G1* and *phzA2-G2*. They are 98.3% identical to each other on the DNA level, so that we could not unambiguously map the transcripts to either of the two clusters. Hence, the differential expression of *phzC-G* could not be analyzed. The only differences in the two *phzA-G* clusters occur in the first two gene couples *phzA1B1* and *phzA2B2* (Mavrodi et al. 2001). The phenazine precursor PCA can be transformed into pyocyanine, the best studied phenazine of PA, by PhzM and PhzS, which occur as single copies in the PA genome. Other phenazines are also based on PCA, e.g. 1-hydroxy-phenazine

generated by PhzS alone, or phenazine-1-carboxamide synthesized by PhzH (Mavrodi et al. 2001; Mentel et al. 2009; Pierson and Pierson 2010; Jayaseelan et al. 2014). The PQS system of PA controls the synthesis of phenazines (Dubern and Diggle 2008). The toxic effect of phenazines meinly derives from their ability to redox cycle (Mazzola et al. 1992; Mavrodi et al. 2001; Gibson et al. 2009; Morales et al. 2010; Jayaseelan et al. 2014). They can form reactive oxygen species (ROS), which interfere with normal cell functions (e.g. the respiratory chain) (Lau et al. 2004). Additionally, PCA and pyocyanine are able to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron, thus increasing the bioavailability of this essential nutrient (Cox 1986; Pierson and Pierson 2010; Cornelis and Dingemans 2013). Under iron limitation, where pyoverdine is increasingly synthesized (Figure 3, 4b+d, S2c+d), the additional secretion of pyocyanine or PCA can be a way to aggravate competition for iron between species. The species that is more efficient in iron scavenging gains a competitive advantage over the other (Weaver and Kolter 2004; Harrison et al. 2008; Leinweber et al. 2017b; Niehus et al. 2017; Sexton et al. 2017). Taken together, our results indicate that PA invests in the synthesis of phenazines under iron limitation in response to danger cues from BC. PA could use these phenzines to mediate inter-specific competition first by harming BC directly through the generation of reactive oxygen species, and second, indirectly by impeding access to iron for BC.

Another toxic compound secreted by PA is hydrogen cyanide (HCN), which is synthesized by the products of the operon *hcnABC*. HCN has been shown to suppress BC growth (Bernier et al. 2016) probably by interfering with the aerobic respiratory chain (Anderson et al. 2010). We found that HCN synthesis genes are increasingly transcribed by PA in iron limited but not in iron rich conditions upon receiving BC derived diffusible cues (Figure 9). HCN synthesis is controlled by the las and rhl QS systems of PA and it is further regulated by environmental factors (Bernier et al. 2016). The transcriptional regulator ANR mediates a drastic increase of the expression of the HCN synthesis genes under microoxyc conditions (Pessi and Haas 2000). Furthermore, the functionality of ANR is iron dependent, since it contains an Fe-S cluster as a cofactor. We showed that the transcription of HCN synthesis genes is downregulated in iron limitation without danger cues from a competing species (Figure 3). However, a threat by a distant competitor leads PA to increase its investment in the HCN synthesis by increasing the transcription of the HCN synthase (Figure 9). These findings further support the hypothesis of the HCN synthesis being strongly regulated by different environmental conditions.

Another way, how PA can defend its ecological niche against competitors is by utilizing specific bactericidal pyocins. In PA we find F, R and S type pyocins that kill susceptible cells of the same or closely related species very effectively (Riley and Wertz 2002). S2, S4 and S5 pyocins, which are upregulated in our study in iron rich but downregulated in iron limited conditions upon growth in BC conditioned medium (Figure 9, Table 1 and 3), kill susceptible cells either by DNAse, tRNAse or pore-

forming activity, respectively (Parret and De Mot 2002). We found the genes encoding the R2 pyocin of PA being upregulated in iron rich and downregulated in iron limited medium upon growth in BC supernatant. The genes encoding PA's F2-pyocin show the same trend, but are mostly non-significantly regulated (Figure 9, Table 1 and 3). S-type pyocins are similar to the *Escherichia coli* colicins, consisting of a toxin- and an immunity-domain that are synthesized together. In contrast to the proteinaceous soluble S-type pyocins, the R- and F-type pyocins are similarly structured to phage tails. They consist of a baseplate with tail fibres, a sheath, and a core for the R-type pyocin and a rod-shaped structure with distal fibres for the F-type pyocin (Michel-Briand and Baysse 2002). The R-type pyocin binds to lipopolysaccharides of its target cells, after which it contracts its sheath and injects its core into the cytosol of the cell. This pore formation leads to the depolarization of the cell envelop and consequently to the death of the target cell (Michel-Briand and Baysse 2002; Köhler et al. 2010). Pyocins can be induced by DNA damage (Matsui et al. 1993), which indicates that the BC supernatant from iron rich conditions could contain DNA damaging compounds. Although, here the expression of pyocin genes is stimulated by BC derived compounds, their effectiveness against other bacterial species is rather inconclusive. Pyocins are usually considered to be narrow-target antibiotics, which are active against closely related bacteria (Riley and Wertz 2002). S2- and S4-pyocin need the FpvA1 ferripyoverdine receptor, and S5-pyocin the FptA ferripyochelin receptor of PA to enter their target cells (Elfarash et al. 2012, 2014). Although BC produces a very similar ferripyochelin receptor, PA cannot kill BC via its S5 pyocin, as shown by Elfarash et al. 2014 Thus, it is questionable to what extent S-type pyocins contribute to the competition between PA and BC. R- and F-pyocins have a somewhat broader target spectrum. Bakkal et al demonstrated that *Burkholderia cepacia* complex species can be inhibited by R- and F-pyocins derived from clinical isolates of PA, thus it is possible that PA uses them to compete against BC (Bakkal et al. 2010).

***Table 3**: Summary of gene expression analysis of PA genes that encode R2- and F2-type pyocins, genes encoding PA's S-type pyocins (S2, S4 and S5) and genes coding for respective immunity proteins. The gene expression was analyzed in PA grown in BC conditioned medium relative to PA conditioned medium in either iron limited or iron rich conditions separately. The table summarizes the Base Mean (number of unique reads corrected for library size across all samples of the two medium treatments), the log2 transformed gene expression fold change (log2FC), the standard error (SE) of the log2FC, and FDR adjusted p-value (padj) of the log2FC. The table contains only genes, of which transcripts could be detected in at least one of the two iron treatments. Entries with purple, green or orange background show genes that are significantly (padj ≤ 0.1) differentially regulated in iron rich, iron limited or in both iron conditions, respectively. Entries written in grey depict genes, whose p-value could not be assigned in one of the two or both iron conditions.*

				Iron limited conditions				Iron rich conditions			
Pyocin Type	**Function**	**Locus Tag**	**Gene Name**	**Base Mean**	**log2FC**	**SE**	**padj**	**Base Mean**	**log2FC**	**SE**	**padj**
S2	toxin	PA1150	pys2	4649.746	-0.696	0.273	0.087	4259.660	0.723	0.173	0.001
S4	toxin	PA3866	PA3866	12090.115	-1.333	0.275	0.000	1122.765	0.923	0.224	0.001
S5	toxin	PA0985	pyoS5	3062.976	-1.814	0.281	0.000	677.441	1.481	0.253	0.000
S2	immunity	PA1151	imm2	6740.152	-0.758	0.277	0.057	10361.742	0.185	0.169	0.478
S5	immunity	PA0984	s5I	39.580	-0.431	0.364	0.582	NA	NA	NA	NA
R2	structural	PA0626	PA0626	70.613	-0.202	0.322	0.838	135.773	1.316	0.272	0.000
		PA0625	PA0625	51.958	-0.152	0.404	0.922	69.993	1.278	0.345	0.002
		PA0624	PA0624	NA	NA	NA	NA	22.298	0.048	0.436	NA
		PA0623	PA0623	220.064	-1.097	0.259	0.001	1068.872	0.844	0.259	0.009
		PA0622	PA0622	1259.146	-1.850	0.245	0.000	5321.022	1.174	0.248	0.000
		PA0621	PA0621	96.299	-0.537	0.273	0.243	112.012	1.291	0.259	0.000
		PA0620	PA0620	224.377	-0.150	0.254	0.850	396.779	1.010	0.256	0.001
		PA0619	PA0619	60.302	-0.995	0.381	0.075	149.467	1.277	0.310	0.001
		PA0618	PA0618	20.175	-0.528	0.439	NA	45.523	0.256	0.341	0.649
		PA0617	PA0617	NA	NA	NA	NA	7.604	0.294	0.511	NA
		PA0616	PA0616	124.111	-0.344	0.261	0.522	351.748	0.931	0.258	0.003
		PA0615	PA0615	102.295	-0.237	0.374	0.837	65.703	0.298	0.318	0.552
F2	structural	PA0633	PA0633	NA	NA	NA	NA	56.085	1.066	0.367	0.022
		PA0635	PA0635	33.693	-0.907	0.451	NA	53.482	1.479	0.344	0.000
		PA0636	PA0636	55.302	-0.474	0.387	0.564	100.022	1.541	0.319	0.000
		PA0637	PA0637	NA	NA	NA	NA	51.429	2.230	0.382	0.000
		PA0638	PA0638	NA	NA	NA	NA	32.648	0.204	0.421	NA
		PA0639	PA0639	32.374	0.100	0.374	NA	67.260	0.831	0.383	0.105
		PA0640	PA0640	20.154	0.667	0.451	NA	28.186	1.558	0.406	NA
		PA0641	PA0641	226.319	-0.500	0.291	0.337	586.640	0.929	0.239	0.001
		PA0642	PA0642	30.499	0.105	0.440	NA	38.650	0.179	0.413	NA
		PA0643	PA0643	97.177	-0.197	0.370	0.873	77.498	0.545	0.339	0.257
		PA0644	PA0644	62.634	-0.148	0.351	0.913	54.014	0.897	0.334	0.037
		PA0645	PA0645	22.871	0.843	0.429	NA	21.884	-0.502	0.473	NA

				Iron limited conditions				Iron rich conditions			
Pyocin Type	**Function**	**Locus Tag**	**Gene Name**	**Base Mean**	**log2FC**	**SE**	**padj**	**Base Mean**	**log2FC**	**SE**	**padj**
		PA0646	PA0646	136.006	-0.181	0.360	0.882	214.173	0.334	0.270	0.405
		PA0647	PA0647	17.710	0.225	0.468	NA	19.040	0.478	0.485	NA
		PA0648	PA0648	17.087	-1.184	0.462	NA	14.741	1.302	0.497	NA

Bacterial competition could also be mediated by secreted hydrolyzing enzymes that degrade organic matter either derived from multicellular organisms or other bacteria (Kögel-Knabner 2002; Willsey et al. 2015). Hydrolyzing enzymes could directly harm other bacteria (i.e. perturbing lipid membranes), accelerate nutrient uptake for the enzyme-secreting strain, or they could establish protection from antagonistic compounds, like proteinaceous toxins or lipid membrane vesicles derived from competing bacterial species (Hibbing et al. 2010; Schwechheimer and Kuehn 2015). But secreted hydrolyzing enzymes form PA are mainly considered to be virulence factors that degrade tissue to free nutrients and influence the immune response (Döring et al. 1988; Cahan et al. 2001; Barker et al. 2004; Vance et al. 2004; Vasil 2006; Kida et al. 2008; Laarman et al. 2012; Banthiya et al. 2016). The majority of PA's hydrolyzing enzymes is secret by the T1SS and T2SS (Filloux 2011).

Under our tested conditions, we found three genes encoding lipolytic enzymes whose transcription was altered, when PA grew in the presence of BC-secreted cues. Under both iron availabilities, the expression of *plcB* is downregulated. *LipA* transcription is upregulated in iron limited medium, and *loxA* transcription is upregulated in iron replete medium (Figure 9). LipA is PA's main extracellular lipase, which binds alginate, so that it stays in close proximity to the producing cell (Stuer et al. 1986; Tielen et al. 2013), where it hydrolizes a broad range of lipdis to produce free fatty acids (Bofill et al. 2010). The phospholipase PlcB and the lipoxygenase LoxA have been shown to be important in the host context. PlcB is a virulence factor that is important in the early colonization of the cystic fibrosis lung, where it facilitates chemotactic twitching towards membrane phospholipids to degrade them (Barker et al. 2004; Vasil 2006; Miller et al. 2008). One of these phospholipids, phosphatidylethanolamine is also a main phospholipid in membranes of gram-negative bacteria (Epand et al. 2007). Hence, PlcB potentially facilitates bacterial membrane degradation to take up nutrients. LoxA specifically degrades unsaturated fatty acids, which are rarely synthesized by bacteria but by organisms of higher taxa (Vance et al. 2004; Starkey et al. 2009; Deschamps et al. 2016). Thus, it is also thought to play an important role in the interactions with eukaryotes i.e. by oxygenating mammalian membrane phospholipids, by inducing hemolysis or by silencing the immune response (Banthiya et al. 2016).

When PA grew in BC conditioned medium, it either downregulated or did not significantly change the expression of genes encoding its secreted proteases, except for *impA*, which is upregulated in iron rich conditions (Figure 9). *ImpA* encodes a metalloprotease, which is known for its immune-modulating activity (Bardoel et al. 2012).

The expression of PA's phosphatase genes *lapA* and *phoA* is not significantly changed in our experimental setting (Figure 9). The two alkaline phosphatases, LapA and PhoA, are secreted by the Hxc- and Xcp-T2SS, respectively, and take up extracellular phosphate as a nutrient. We stated above, that gene transcripts encoding the Hxc-T2SS could not be detected under our settings. This finding corresponds to the expression pattern of PA's phosphatases, which indicates that phosphate is no limiting factor in our tested media conditions.

We also looked into other enzymes, secreted by PA that have a clear function to defect target cells of organisms of higher taxa. The gene expression pattern of the T3SS toxins ExoS, ExoT and ExoY has not significantly changed in PA upon growth in BC supernatant. These results correspond to the gene expression of the T3SS structural genes, indicating that this secretion system is not expressed in a non-host context (compare Figure 8 and 9).

The expression of *aprX* and *aprA* is downregulated in PA under iron limitation and upon growth in BC supernatant (Figure 9). This corresponds to the downregulation of the genes that encode the Apr-T1SS (*aprDEF*) (Figure 8). This secretion system specifically secretes the two enzymes AprA and AprX. While AprA constitutes an alkaline metalloproteinase, the function of AprX in PA is still not understood (Duong et al. 2001).

The expression of t*oxA* is downregulated upon growth of PA in BC conditioned medium under iron limitation, but non-significantly regulated in iron rich medium (Figure 9). *ToxA* encodes Exotoxin A that is secreted by the Xcp-T2SS. Exotoxin A specifically binds to the eukaryotic elongation factor-2 and thereby inhibits the protein synthesis (Iglewski et al. 1977).The *toxA* transcription is positively controlled by the anti-sigma factor PvdS, that also regulates pyoverdine and Protease IV (encoded by *prpL*) synthesis (Ochsner et al. 1996; Wilderman et al. 2001; Lamont et al. 2002). The gene regulation of these three PA traits (pyoverdine, Exotoxin A and protease IV) in response to BC derived diffusible cues coincides in iron limitation, where the expression of the respective genes is reduced. But pyoverdine synthesis genes are upregulated in iron rich conditions, where the *prpL and toxA* transcription do not change significantly (compare Figure 5 and 9). This indicates that other regulatory mechanisms might play a role that circumvent PvdS as a transcriptional regulator.

In contrast to the broad functional spectrum of secreted enzymes via T1SS and T2SS, the three types of T6SS (H1-, H2- and H3-T6SS) take on a very specific function in the competition of PA against other

bacteria. The T6SS directly injects effector proteins into target cells, where they deploy their toxic effects. In our experiments we found that the supernatant from PA's competitor BC exerts different transcriptional effects on the T6SS effectors of PA, depending on the iron availability of the medium. In iron limited conditions the genes for T6SS secreted enzymes are generally downregulated, while under iron rich conditions they are generally upregulated, though not all regulations are significant (Figure 9, Table 1 and 2). We found that the genes encoding the different T6SS effector proteins are regulated together with the structural genes for the respective T6SS machineries. That means, that the gene expression of two H1-T6SS secreted enzymes, Tse3 and Tse5, is significantly upregulated by PA in iron limited conditions when it grew in BC supernatant. The genes *Tle3* and *pldA* (encoding phospholipase D) that encode H2-T6SS secreted enzymes, are primarily transcribed upon contact with diffusible BC signals in iron replete medium. The *PldA* transcription is significantly downregulated by PA in iron limited conditions upon growth in BC supernatant. Gene expression of H3-T6SS secreted enzymes is not significantly altered in PA upon receiving competitive cues from BC (Table 2). *Tse3* and *tse5*, whose gene products are secreted by the bacteria-specific H1-T6SS, are bactericidal. Tse3 acts as a muramidase, degrading peptidoglycan of bacterial cell walls. Tle3 and PldA show lipolytic activity, thus damaging the cell wall integrity of the prokaryotic or eukaryotic target cell (Sana et al. 2016). One of the substrates of PldA is phosphatidylethanolamine, the major phospholipid in bacterial membranes (Russell et al. 2013). Besides their role in inter-bacterial competition, the phospholipases D (PldA and PldB), secreted by the H2- and H3-T6SS, are important players for PA pathogenesis. They mediate cross-kingdom interactions by binding to the actin cytoskeleton of mammalian cells and thus facilitate the internalization of PA into epithelial host cells (Sana et al. 2016). PA usually co-expresses effector and cognate immunity genes to prevent self-intoxication (Chen et al. 2015; Alteri and Mobley 2016; Sana et al. 2016). However, in our experimental setup we found only the immunity genes *tli5a* and *tli3* to be significantly regulated in the same way as their H2-T6SS lipolytic effectors PldA and Tle3, respectively. Gene transcripts for H1-T6SS immunity proteins were detected only at low numbers, in contrast to the respective toxin genes, so that no significant change in expression levels could be determined for them (Table 2). Taken together, PA initiates the expression of H1- and H2-T6SS structural genes and of the cognate secreted enzymes, when it receives diffusible competition cues through the BC supernatant. This could be a mechanism of PA to prepare for an encounter with a yet distant competitor. However, this scenario strongly depends on a high iron availability of the medium.

Discussion

Bacteria live in natural multispecies communities with a high degree of inter-specific competition for space and nutrients (Hibbing et al. 2010). Therefore, it is beneficial to be able to sense the presence of a competitor in order to start a counter-attack or switch to defense mode (Cornforth and Foster 2013). The classical view towards competition sensing is that bacteria react to direct contact with other bacterial cells. However, it is becoming apparent, that other mechanisms are just as important. In nature, bacteria grow in habitats with more or less spatial structure that facilitate diffusion of secreted compounds (relative to their physical size) to different degrees. Westhoff et al. have reviewed examples, where bacteria react to diffusible cues from other bacteria and change their phenotype in response, without having actual physical contact with each other (Westhoff et al. 2017). These mechanisms can be seen as means for bacteria to switch to a defense or an attack mode to be prepared for an upcoming assault.

Here, we used RNA sequencing to analyze PA's initial response to competitive cues produced by BC. We found that the diffusible cues from BC induce a phenotypical and global transcriptional change. We found that PA employs a multivariate response, significantly regulating the gene expression in all our mechanisms of interest. But which mechanisms are regulated and in which direction depended strongly on the iron availability of the medium. Cues derived from PA's competitor BC lead to the initiation of a toxic attack under iron limited conditions involving HCN and phenazine synthesis and the lipase LipA. Under iron rich conditions however, PA induced a broader response. Here, we found that structural flagella genes were increasingly expressed, as well as genes responsible for nutrient uptake like pyoverdine synthesis and receptor genes, genes for the metalloprotease ImpA and the lipoxygenase LoxA. Under high iron availability, PA further upregulates the expression of genes coding for antibacterial strategies like H1- and H2-T6SS and the respective secreted enzymes, CDI related T5SS and pyocins. Only few PA traits were regulated upon growth in BC conditioned medium independently from the iron availability. Only the genes encoding the HHQ synthesis pathway, a PQS precursor with QS regulatory and bacteriostatic capacities, were increasingly expressed after growth in BC supernatant under both tested iron availabilities. The expression of genes coding for AHL based QS systems, biofilm formation, the synthesis pathway of PA's secondary siderophore pyochelin, and the T3SS and respective secreted enzymes are either downregulated or not affected by the BC supernatant in either iron rich or limited medium.

One reason for the diverse gene regulation between high and low iron availability might be, that PA enzymes and regulators, which use iron as a co-factor, might not be functioning under iron restriction. PA channels the limited iron resources to other mechanisms that might be more important under the given circumstances (Andrews et al. 2003).

By using RNA sequencing, we were able to analyze PA's response to BC derived cues on the transcriptional level. However, this response could be further adjusted post-transcriptionally and post-translationally, so that the final competitive strategy of PA against BC could diverge from the transcriptional response we observed in our experiments. Furthermore, our data shows PA's transcriptional response to BC cues at a time point during mid to late exponential phase. However, many PA traits are subject to temporal changes. For example, while we measured the cumulative level of *de novo* synthesized pyoverdine over 48 hours in PA monocultures grown in either its own supernatant or the competitor (BC) supernatant mixed with fresh CAA medium, or grown in fresh CAA medium only (Figure S2 and S3), the transcription of the pyoverdine synthesis genes was analyzed at a certain time point during growth (Figure 5). Because the synthesis of pyoverdine is time dependent, the two measurements (overall *de novo* pyoverdine level and gene expression) do not necessarily correspond to each other. This is probably the case in iron limited conditions, where the *de novo* pyoverdine level is higher in PA grown in BC supernatant than in its own supernatant (Figure S2 and S3), but the pyoverdine synthesis genes are downregulated in PA grown in BC supernatant relative to its own supernatant during mid to late exponential phase (Figure 5).

In order to initiate a competitive response, bacteria need to be able to sense the presence of competitors. Cornforth and Foster distinguish two possible ways to do so: first, indirect competition sensing through environmental changes (i.e. nutrient limitation or oxidative stress) or cell damage, and second direct competition sensing via competitor-derived cues (Cornforth and Foster 2013). These cues can be diffusible signals like secreted enzymes, QS molecules or toxins, which are reviewed by Westhoff et al. 2017.In our experiments, PA receives only diffusible cues from BC and cannot sense the competition through direct cell-cell contact. Thereby, PA could respond to a single cue or to multiple cues simultaneously that are present in the spent BC culture supernatant. However, the exact nature of the BC derived cue that is sensed by PA is still unclear. I will now give a short overview of possible mechanisms, how PA could receive the presence of BC in its proximity. One possible danger cue could be BC derived QS molecules. It has been shown that bacteria can exchange QS signals across species boarders. This so called "inter-specific cross talk" has been shown to be relevant for AHLs and short fatty acids, also termed diffusible signal factors (DSF), which are used by many gram-negative bacteria (Fuqua et al. 2001; Wang et al. 2003; LeRoux et al. 2015b; Ryan et al. 2015). AHLs and DSF could play a role in competition sensing in PA, as it has been shown that they can be exchanged between PA and *Burkholderia* species (Riedel et al. 2001; Deng et al. 2013). BC secretes the short fatty acid signal cis-2-dodecanoic acid (*Burkholderia* diffusible signal factor, BDSF), as QS signaling molecule, which was shown to impact the gene expression in PA. It affects many of the PA traits that were also regulated in our experiments, like QS, proteases or pyocyanine synthesis indicating that the BDSF signal

might contribute to competition sensing of PA in our settings (Davies and Marques 2009; Deng et al. 2013). Other danger cues for PA could be fragments of BC membranes or outer membrane vesicles that are common among gram-negative bacteria (Allan et al. 2003; Jan 2017). The membrane fragments contain peptidoglycan, which is a main structural compound of bacterial membranes. Peptidoglycan has been shown to lead to an upregulation of the pyocyanine production in PA (Korgaonkar et al. 2013). Given the multiple ways how bacterial toxins can compromise bacterial cell integrity it is also important to note, that PA responds to diffusible signals of lysed kin cells by upregulating its H1-T6SS (LeRoux et al. 2015a). Since PA can live in many different habitats and encounters various polymicrobial communities in these habitats, it is conceivable that PA employs various ways to perceive and outcompete competitors like BC (Weaver and Kolter 2004; Harrison 2007; LiPuma 2010).

To our knowledge, this is the first global study of PA's transcriptional response to diffusible danger signals derived from BC, and that also considers different nutritional conditions. We used previous investigations on narrower sets of competition responses to define our groups of interest, which contain mechanisms that mediate bacterial competition, or are affected by it. We focused mainly on mechanisms whose gene products directly affect the competition between PA and BC. We did not analyze competition sensing and signal transduction pathways in detail, because these pathways are often not regulated on the transcriptional level by modulating the amount of mRNA. But they are rather regulated by enzyme activity, like kinases and phosphatases, that switch on/off certain signal transductions via phosphorylation and dephosphorylation. Two future courses of research could be taken from here. First, it would be interesting to find out which diffusible cues exactly confer competition to PA and what molecular mechanisms modulate the response to diffusible competitive cues in PA. Second, one could investigate if the response on the transcriptional level directly corresponds to the final amount of the effectors, or if there are other regulatory processes involved on the post-transcriptional and post-translational level that further adjust PA's competition response.

Literature

Allan, N. D., C. Kooi, P. A. Sokol, and T. J. Beveridge. 2003. Putative virulence factors are released in association with membrane vesicles from *Burkholderia cepacia*. Can. J. Microbiol. 49:613–624.

Alteri, C. J., and H. L. T. T. Mobley. 2016. The Versatile Type VI Secretion System. Microbiol. Spectr. 4:337–356.

Anderson, R. D., L. F. Roddam, S. Bettiol, K. Sanderson, and D. W. Reid. 2010. Biosignificance of bacterial cyanogenesis in the CF lung. J. Cyst. Fibros. 9:158–164.

Andrews, S. C., A. K. Robinson, and F. Rodríguez-Quiñones. 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev. 27:215–237.

Aujoulat, F., F. Roger, A. Bourdier, A. Lotthé, B. Lamy, H. Marchandin, and E. Jumas-Bilak. 2012. From environment to man: genome evolution and adaptation of human opportunistic bacterial pathogens. Genes. 3:191–232.

Bakkal, S., S. M. Robinson, C. L. Ordonez, D. A. Waltz, and M. A. Riley. 2010. Role of bacteriocins in mediating interactions of bacterial isolates taken from cystic fibrosis patients. Microbiology 156:2058–2067.

Ball, G., É. Durand, A. Lazdunski, and A. Filloux. 2002. A novel type II secretion system in Pseudomonas aeruginosa. Mol. Microbiol. 43:475–485.

Banthiya, S., J. Kalms, E. Galemou Yoga, I. Ivanov, X. Carpena, M. Hamberg, H. Kuhn, and P. Scheerer. 2016. Structural and functional basis of phospholipid oxygenase activity of bacterial lipoxygenase from Pseudomonas aeruginosa. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1861:1681–1692.

Bardoel, B. W., D. Hartsink, M. M. Vughs, C. J. C. de Haas, J. A. G. van Strijp, and K. P. M. van Kessel. 2012. Identification of an immunomodulating metalloprotease of Pseudomonas aeruginosa (IMPa). Cell. Microbiol. 14:902–913.

Barker, A. P., A. I. Vasil, A. Filloux, G. Ball, P. J. Wilderman, and M. L. Vasil. 2004. A novel extracellular phospholipase C of Pseudomonas aeruginosa is required for phospholipid chemotaxis. Mol. Microbiol. 53:1089–1098.

Basler, M., B. T. Ho, and J. J. Mekalanos. 2013. Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. Cell 152:884–894.

Basler, M., and J. J. Mekalanos. 2012. Type 6 Secretion Dynamics Within and Between Bacterial Cells. Science 337:815.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a pratical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. A 57:289–300.

Bernier, S. P., M. L. Workentine, X. Li, N. A. Magarvey, G. A. O'Toole, and M. G. Surette. 2016. Cyanide toxicity to Burkholderia cenocepacia is modulated by polymicrobial communities and environmental factors. Front. Microbiol. 7:725.

Bleves, S., V. Viarre, R. Salacha, G. P. F. Michel, A. Filloux, and R. Voulhoux. 2010. Protein secretion systems in Pseudomonas aeruginosa: A wealth of pathogenic weapons. Int. J. Med. Microbiol. 300:534–543.

Blumer, C., and D. Haas. 2000. Iron regulation of the hcnABC genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in Pseudomonas fluorescens CHA0. Microbiology 146:2417–2424.

Bofill, C., N. Prim, M. Mormeneo, A. Manresa, F. I. Javier Pastor, and P. Diaz. 2010. Differential behaviour of Pseudomonas sp. 42A2 LipC, a lipase showing greater versatility than its counterpart LipA. Biochimie 92:307–316.

Bollinger, N., D. J. Hassett, B. H. Iglewski, J. W. Costerton, and T. R. McDermott. 2001. Gene Expression in Pseudomonas aeruginosa: Evidence of Iron Override Effects on Quorum Sensing and Biofilm-Specific Gene Regulation. J. Bacteriol. 183:1990–1996.

Borlee, B. R., A. D. Goldman, K. Murakami, R. Samudrala, D. J. Wozniak, and M. R. Parsek. 2010. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol. Microbiol. 75:827–42.

Burrows, L. L. 2012. *Pseudomonas aeruginosa* Twitching Motility: Type IV Pili in Action. Annu. Rev. Microbiol. 66:493–520.

Cahan, R., I. Axelrad, M. Safrin, D. E. Ohman, and E. Kessler. 2001. A secreted aminopeptidase of Pseudomonas aeruginosa. Identification, primary structure, and relationship to other aminopeptidases. J. Biol. Chem. 276:43645–52.

Chen, L., Y. Zou, P. She, and Y. Wu. 2015. Composition, function, and regulation of T6SS in Pseudomonas aeruginosa. Microbiol. Res. 172:19–25.

Cianciotto, N. P., and R. C. White. 2017. Expanding Role of Type II Secretion in Bacterial Pathogenesis and Beyond. Infect. Immun. 85:e00014-17.

Coenye, T., and P. Vandamme. 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ. Microbiol. 5:719–729.

Cornelis, P. 2010. Iron uptake and metabolism in pseudomonads. Appl. Microbiol. Biotechnol. 86:1637–1645.

Cornelis, P., and J. Dingemans. 2013. Pseudomonas aeruginosa adapts its iron uptake strategies in function of the type of infections. Front. Cell. Infect. Microbiol. 3:1–7.

Cornforth, D. M., and K. R. Foster. 2013. Competition sensing: the social side of bacterial stress responses. Nat. Rev. Microbiol. 11:285–293.

Cox, C. D. 1986. Role of pyocyanin in the acquisition of iron from transferrin. Infect. Immun. 52:263–270.

Curtis, T. P., W. T. Sloan, and J. W. Scannell. 2002. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. U. S. A. 99:10494–10499.

Davies, D. G., and C. N. H. Marques. 2009. A fatty acid messenger is responsible for inducing dispersion in microbial biofilms. J. Bacteriol. 191:1393–403.

Deng, Y., C. Boon, S. Chen, A. Lim, and L. Zhang. 2013. Cis -2-dodecenoic acid signal modulates virulence of Pseudomonas aeruginosa through interference with quorum sensing systems and T3SS. BMC Microbiol. 13:231.

Deschamps, J. D., A. F. Ogunsola, J. B. Jameson, A. Yasgar, B. A. Flitter, C. J. Freedman, et al. 2016. Biochemical and Cellular Characterization and Inhibitor Discovery of Pseudomonas aeruginosa 15-Lipoxygenase. Biochemistry 55:3329–3340.

Diggle, S. P., S. Matthijs, V. J. Wright, M. P. Fletcher, S. R. Chhabra, I. L. Lamont, et al. 2007. The Pseudomonas aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment. Chem. Biol. 14:87–96.

Döring, G., M. Pfestorf, K. Botzenhart, and M. A. Abdallah. 1988. Impact of proteases on iron uptake of Pseudomonas aeruginosa pyoverdin from transferrin and lactoferrin. Infect. Immun. 56:291–293.

Dubern, J.-F., and S. P. Diggle. 2008. Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species. Mol. Biosyst. 4:822–888.

Dumas, Z., A. Ross-Gillespie, and R. Kümmerli. 2013. Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. Proc. R. Soc. B 280:20131055.

Duong, F., E. Bonnet, V. Géli, A. Lazdunski, M. Murgier, and A. Filloux. 2001. The AprX protein of Pseudomonas aeruginosa: a new substrate for the Apr type I secretion system. Gene 262:147–153.

Elfarash, A., J. Dingemans, L. Ye, A. A. Hassan, M. Craggs, C. Reimmann, M. S. Thomas, and P. Cornelis. 2014. Pore-forming pyocin S5 utilizes the FptA ferripyochelin receptor to kill Pseudomonas aeruginosa. Microbiology 160:261–269.

Elfarash, A., Q. Wei, and P. Cornelis. 2012. The soluble pyocins S2 and S4 from Pseudomonas aeruginosa bind to the same FpvAI receptor. Microbiologyopen 1:268–75.

Epand, R. F., P. B. Savage, and R. M. Epand. 2007. Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins). Biochim. Biophys. Acta - Biomembr. 1768:2500–2509.

Fang, Y., G. Xie, M. Lou, B. Li, and I. Muhammad. 2011. Diversity analysis of Burkholderia cepacia complex in the water bodies of West Lake, Hangzhou, China. J. Microbiol. 49:309–314.

Faure, L. M., S. Garvis, S. de Bentzmann, and S. Bigot. 2014. Characterization of a novel two-partner secretion system implicated in the virulence of Pseudomonas aeruginosa. Microbiology 160:1940–1952.

Fazli, M., H. Almblad, M. L. Rybtke, M. Givskov, L. Eberl, and T. Tolker-Nielsen. 2014. Regulation of biofilm formation in Pseudomonas and Burkholderia species. Environ. Microbiol. 16:1961–1981.

Filloux, A. 2011. Protein secretion systems in Pseudomonas aeruginosa: An essay on diversity, evolution, and function. Front. Microbiol. 2:1–21.

Fuqua, C., M. R. Parsek, and E. P. Greenberg. 2001. Regulation of Gene Expression by Cell-to-Cell Communication: Acyl-Homoserine Lactone Quorum Sensing. Annu. Rev. Genet. 35:439–468.

Ghysels, B., B. Thi Min Dieu, S. A. Beatson, J.-P. Pirnay, U. A. Ochsner, M. L. Vasil, and P. Cornelis. 2004. FpvB, an alternative type I ferripyoverdine receptor of Pseudomonas aeruginosa. Microbiology 150:1671–1680.

Gibson, J., A. Sood, and D. A. Hogan. 2009. Pseudomonas aeruginosa-Candida albicans interactions: localization and fungal toxicity of a phenazine derivative. Appl. Environ. Microbiol. 75:504–13.

Girard, G., and G. V Bloemberg. 2008. Central role of quorum sensing in regulating the production of pathogenicity factors in *Pseudomonas aeruginosa*. Future Microbiol. 3:97–106.

Giraud, C., and S. de Bentzmann. 2012. Inside the complex regulation of Pseudomonas aeruginosa chaperone usher systems. Environ. Microbiol. 14:1805–1816.

Gotschlich, A., B. Huber, O. Geisenberger, A. Togl, A. Steidle, K. Riedel, et al. 2001. Synthesis of multiple N-acylhomoserine lactones is wide-spread among the members of the Burkholderia cepacia complex. Syst. Appl. Microbiol. 24:1–14.

Graff, J. R., S. R. Forschner-Dancause, S. Menden-Deuer, R. A. Long, and D. C. Rowley. 2013. Vibrio cholerae Exploits Sub-Lethal Concentrations of a Competitor-Produced Antibiotic to Avoid Toxic Interactions. Front. Microbiol. 4:8.

Hall-Stoodley, L., J. W. Costerton, and P. Stoodley. 2004. Bacterial biofilms: from the Natural environment to infectious diseases. Nat. Rev. Microbiol. 2:95–108.

Hariprasad, P., S. Chandrashekar, S. B. Singh, and S. R. Niranjana. 2014. Mechanisms of plant growth promotion and disease suppression by *Pseudomonas aeruginosa* strain 2apa. J. Basic Microbiol. 54:792–801.

Harrison, F. 2007. Microbial ecology of the cystic fibrosis lung. Microbiology 153:917–923.

Harrison, F., J. Paul, R. C. Massey, and A. Buckling. 2008. Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. ISME J. 2:49–55.

Hayes, C. S., S. K. Aoki, and D. A. Low. 2010. Bacterial Contact-Dependent Delivery Systems. Annu. Rev. Genet. 44:71–90.

Hibbing, M. E., C. Fuqua, M. R. Parsek, and S. B. Peterson. 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8:15–22.

Hoffman, L. R., D. A. D'Argenio, M. J. MacCoss, Z. Zhang, R. A. Jones, and S. I. Miller. 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature 436:1171–1175.

Hölscher, T., B. Bartels, Y.-C. Lin, R. Gallegos-Monterrosa, A. Price-Whelan, R. Kolter, L. E. P. Dietrich, and Á. T. Kovács. 2015. Motility, Chemotaxis and Aerotaxis Contribute to Competitiveness during Bacterial Pellicle Biofilm Development. J. Mol. Biol. 427:3695–3708.

Iglewski, B. H., P. V Liu, and D. Kabat. 1977. Mechanism of action of Pseudomonas aeruginosa exotoxin Aiadenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. 15:138–44.

Imberty, A., M. Wimmerová, E. P. Mitchell, and N. Gilboa-Garber. 2004. Structures of the lectins from Pseudomonas aeruginosa: insights into the molecular basis for host glycan recognition. Microbes Infect. 6:221–228.

Imperi, F., F. Tiburzi, and P. Visca. 2009. Molecular basis of pyoverdine siderophore recycling in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 106:20440–20445.

Jan, A. T. 2017. Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. Front. Microbiol. 8:1053.

Jayaseelan, S., D. Ramaswamy, and S. Dharmaraj. 2014. Pyocyanin: production, applications, challenges and new insights. World J. Microbiol. Biotechnol. 30:1159–1168.

Jones, C., L. Allsopp, J. Horlick, H. Kulasekara, and A. Filloux. 2013. Subinhibitory Concentration of Kanamycin Induces the Pseudomonas aeruginosa type VI Secretion System. PLoS One 8:e81132.

Joshi, F., G. Archana, and A. Desai. 2006. Siderophore cross-utilization amongst rhizospheric bacteria and the role of their differential affinities for Fe3+ on growth stimulation under iron-limited conditions. Curr. Microbiol. 53:141–147.

Kahm, M., G. Hasenbrink, H. Lichtenberg-Fraté, J. Ludwig, and M. Kschischo. 2010. grofit: Fitting Biological Growth Curves with R. J. Stat. Softw. 33:1–21.

Kaiser, D. 2007. Bacterial swarming: a re-examination of cell-movement patterns. Curr. Biol. 17:R561–R570.

Kaplan, J. B., E. A. Izano, P. Gopal, M. T. Karwacki, S. Kim, J. L. Bose, K. W. Bayles, and A. R. Horswill. 2012. Low levels of β-lactam antibiotics induce extracellular DNA release and biofilm formation in Staphylococcus aureus. MBio 3:e00198-12.

Kida, Y., Y. Higashimoto, H. Inoue, T. Shimizu, and K. Kuwano. 2008. A novel secreted protease from Pseudomonas aeruginosa activates NF-κB through protease-activated receptors. Cell. Microbiol. 10:1491–1504.

Kim, S.-K., and J.-H. Lee. 2016. Biofilm dispersion in Pseudomonas aeruginosa. J. Microbiol. 54:71–85.

Kögel-Knabner, I. 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. Soil Biol. Biochem. 34:139–162.

Köhler, T., V. Donner, and C. van Delden. 2010. Lipopolysaccharide as shield and receptor for R-pyocin-mediated killing in Pseudomonas aeruginosa. J. Bacteriol. 192:1921–1928.

Konovalova, A., and L. Søgaard-Andersen. 2011. Close encounters: contact-dependent interactions in bacteria. Mol. Microbiol. 81:297–301.

Korgaonkar, A., U. Trivedi, K. P. Rumbaugh, and M. Whiteley. 2013. Community surveillance enhances Pseudomonas aeruginosa virulence during polymicrobial infection. Proc. Natl. Acad. Sci. U. S. A. 110:1059–1064.

Kümmerli, R., and S. P. Brown. 2010. Molecular and regulatory properties of a public good shape the evolution of cooperation. Proc. Natl. Acad. Sci. U. S. A. 107:18921–18926.

Kümmerli, R., N. Jiricny, L. S. Clarke, S. A. West, and A. S. Griffin. 2009. Phenotypic plasticity of a cooperative behaviour in bacteria. J. Evol. Biol. 22:589–598.

Laarman, A. J., B. W. Bardoel, M. Ruyken, J. Fernie, F. J. Milder, J. A. G. van Strijp, and S. H. M. Rooijakkers. 2012. Pseudomonas aeruginosa Alkaline Protease Blocks Complement Activation via the Classical and Lectin Pathways. J. Immunol. 188:386–393.

Lamont, I. L., P. A. Beare, U. Ochsner, A. I. Vasil, and M. L. Vasil. 2002. Siderophore-mediated signaling regulates virulence factor production in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 99:7072–7077.

Lamont, I. L., and L. W. Martin. 2003. Identification and characterization of novel pyoverdine synthesis genes in Pseudomonas aeruginosa. Microbiology 149:833–842.

Lau, G. W., D. J. Hassett, H. Ran, and F. Kong. 2004. The role of pyocyanin in Pseudomonas aeruginosa infection. Trends Mol. Med. 10:599–606.

Lee, J., and L. Zhang. 2015. The hierarchy quorum sensing network in Pseudomonas aeruginosa. Protein Cell 6:26–41.

Leinweber, A., R. Fredrik Inglis, and R. Kümmerli. 2017a. Cheating fosters species co-existence in well-mixed bacterial communities. ISME J. 11:1179–1188.

Leinweber, A., M. Weigert, and R. Kümmerli. 2017b. The bacterium Pseudomonas aeruginosa senses and gradually responds to inter-specific competition for iron. *In Preparation*

Lenney, W., and F. J. Gilchrist. 2011. Pseudomonas aeruginosa and cyanide production. Eur. Respir. J. 37:482–483.

LeRoux, M., J. A. De Leon, N. J. Kuwada, A. B. Russell, D. Pinto-Santini, R. D. Hood, et al. 2012. Quantitative single-cell characterization of bacterial interactions reveals type VI secretion is a double-edged sword. Proc. Natl. Acad. Sci. U. S. A. 109:19804–9.

LeRoux, M., R. L. Kirkpatrick, E. I. Montauti, B. Q. Tran, S. B. Peterson, B. N. Harding, et al. 2015a. Kin cell lysis is a danger signal that activates antibacterial pathways of Pseudomonas aeruginosa. Elife 4:1–25.

LeRoux, M., S. B. Peterson, and J. D. Mougous. 2015b. Bacterial danger sensing. J. Mol. Biol. 427:3744–53.

Li, M., I. Le Trong, M. A. Carl, E. T. Larson, S. Chou, J. A. De Leon, S. L. Dove, R. E. Stenkamp, and J. D. Mougous. 2012. Structural basis for type VI secretion effector recognition by a cognate immunity protein. PLoS Pathog. 8:e1002613.

LiPuma, J. J. 2010. The Changing Microbial Epidemiology in Cystic Fibrosis. Clin. Microbiol. Rev. 23:299–323.

Liu, Y., M. Lardi, A. Pedrioli, L. Eberl, and G. Pessi. 2017. NtrC-dependent control of exopolysaccharide synthesis and motility in Burkholderia cenocepacia H111. PLoS One 12:e0180362.

Loutet, S. A., and M. A. Valvano. 2010. A decade of Burkholderia cenocepacia virulence determinant research. Infect. Immun. 78:4088–100.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550.

Mah, T.-F. C., and G. A. O'Toole. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9:34–39.

Maravic, A., M. Skocibusic, M. Sprung, I. Samanic, J. Puizina, and M. Pavela-Vrancic. 2012. Occurrence and antibiotic susceptibility profiles of Burkholderia cepacia complex in coastal marine environment. Int. J. Environ. Health Res. 22:531–542.

Marr, A. K., J. Overhage, M. Bains, and R. E. W. Hancock. 2007. The Lon protease of Pseudomonas aeruginosa is induced by aminoglycosides and is involved in biofilm formation and motility. Microbiology 153:474–482.

Matsui, H., Y. Sano, H. Ishihara, and T. Shinomiya. 1993. Regulation of pyocin genes in Pseudomonas aeruginosa by positive (prtN) and negative (prtR) regulatory genes. J. Bacteriol. 175:1257–63.

Mavrodi, D. V, R. F. Bonsall, S. M. Delaney, M. J. Soule, G. Phillips, and L. S. Thomashow. 2001. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from Pseudomonas aeruginosa PAO1. J. Bacteriol. 183:6454–6465.

Mazzola, M., R. J. Cook, L. S. Thomashow, D. M. Weller, and L. S. Pierson. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. 58:2616–24.

McKenney, D., K. E. Brown, and D. G. Allison. 1995. Influence of Pseudomonas aeruginosa exoproducts on virulence factor production in Burkholderia cepacia: evidence of interspecies communication. J. Bacteriol. 177:6989–6992.

Mellbye, B., and M. Schuster. 2014. A physiological framework for the regulation of quorum-sensing-dependent public goods in Pseudomonas aeruginosa. J. Bacteriol. 196:1155–1164.

Mentel, M., E. G. Ahuja, D. V. Mavrodi, R. Breinbauer, L. S. Thomashow, and W. Blankenfeldt. 2009. Of Two Make One: The Biosynthesis of Phenazines. ChemBioChem 10:2295–2304.

Mercy, C., B. Ize, S. P. Salcedo, S. de Bentzmann, and S. Bigot. 2016. Functional Characterization of Pseudomonas Contact Dependent Growth Inhibition (CDI) Systems. PLoS One 11:e0147435.

Michel-Briand, Y., and C. Baysse. 2002. The pyocins of Pseudomonas aeruginosa. Biochimie 84:499–510.

Michel, L., A. Bachelard, and C. Reimmann. 2007. Ferripyochelin uptake genes are involved in pyochelin-mediated signalling in Pseudomonas aeruginosa. Microbiology 153:1508–1518.

Miller, R. M., A. P. Tomaras, A. P. Barker, D. R. Voelker, E. D. Chan, A. I. Vasil, and M. L. Vasil. 2008. Pseudomonas aeruginosa twitching motility-mediated chemotaxis towards phospholipids and fatty acids: specificity and metabolic requirements. J. Bacteriol. 190:4038–49.

Mootha, V. K., C. M. Lindgren, K.-F. Eriksson, A. Subramanian, S. Sihag, J. Lehar, et al. 2003. PGC-1α-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. 34:267–273.

Morales, D. K., N. J. Jacobs, S. Rajamani, M. Krishnamurthy, J. R. Cubillos-Ruiz, and D. A. Hogan. 2010. Antifungal mechanisms by which a novel Pseudomonas aeruginosa phenazine toxin kills Candida albicans in biofilms. Mol. Microbiol. 78:1379–1392.

Nair, A. V., N. Joseph, K. Krishna, K. G. Sneha, N. Tom, K. Jangid, and S. Nair. 2015. A comparative study of coastal and clinical isolates of Pseudomonas aeruginosa. Brazilian J. Microbiol. 46:725–734.

Niehus, R., A. Picot, N. M. Oliveira, S. Mitri, and K. R. Foster. 2017. The evolution of siderophore production as a competitive trait. Evolution 71:1443–1455.

Ochsner, U. A., Z. Johnson, I. L. Lamont, H. E. Cunliffe, and M. L. Vasil. 1996. Exotoxin A production in Pseudomonas aeruginosa requires the iron-regulated pvdS gene encoding an alternative sigma factor. Mol. Microbiol. 21:1019–1028.

Ochsner, U. A., P. J. Wilderman, A. I. Vasil, and M. L. Vasil. 2002. GeneChip expression analysis of the iron starvation response in Pseudomonas aeruginosa: identification of novel pyoverdine biosynthesis genes. Mol. Microbiol. 45:1277–1287.

Oliveira, N. M., E. Martinez-Garcia, J. Xavier, W. M. Durham, R. Kolter, W. Kim, and K. R. Foster. 2015. Biofilm formation as a response to ecological competition. PLoS Biol. 13:e1002191.

Parret, A. H. . A., and R. De Mot. 2002. Bacteria killing their own kind: novel bacteriocins of Pseudomonas and other γ-proteobacteria. Trends Microbiol. 10:107–112.

Pessi, G., C. H. Ahrens, H. Rehrauer, A. Lindemann, F. Hauser, H. M. Fischer, and H. Hennecke. 2007. Genome-wide transcript analysis of Bradyrhizobium japonicum bacteroids in soybean root nodules. Mol. Plant-Microbe Interact. 20:1353–1363.

Pessi, G., and D. Haas. 2000. Transcriptional control of the hydrogen cyanide biosynthetic genes hcnABC by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in Pseudomonas aeruginosa. J. Bacteriol. 182:6940–9.

Pierson, L. S., and E. A. Pierson. 2010. Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. Appl. Microbiol. Biotechnol. 86:1659–70.

Poole, K., and G. A. McKay. 2003. Iron acquisition and its control in Pseudomonas aeruginosa: many roads lead to Rome. Front. Biosci. 8:d661-686.

R Development Core Team. 2014. R: A language and environment for statistical computing.

Reen, F. J., M. J. Mooij, L. J. Holcombe, C. M. M. G. P. McGlacken, J. P. Morrissey, and F. O'Gara. 2011. The Pseudomonas quinolone signal (PQS), and its precursor HHQ, modulate interspecies and interkingdom behaviour. Fems Microbiol. Ecol. 77:413–428.

Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, et al. 2001. N -Acylhomoserine-lactone-mediated communication between Pseudomonas aeruginosa and Burkholderia cepacia in mixed biofilms. Microbiology 147:3249–3262.

Riley, M. A., and J. E. Wertz. 2002. Bacteriocin diversity: ecological and evolutionary perspectives. Biochimie 84:357–364.

Rinke, C., P. Schwientek, A. Sczyrba, N. N. Ivanova, I. J. Anderson, J.-F. Cheng, et al. 2013. Insights into the phylogeny and coding potential of microbial dark matter. Nature 499:431–437.

Rusch, D. B., A. L. Halpern, G. Sutton, K. B. Heidelberg, S. Williamson, S. Yooseph, et al. 2007. The Sorcerer II Global Ocean Sampling expedition: northwest Atlantic through eastern tropical Pacific. PLoS Biol. 5:e77.

Russell, A. B., R. D. Hood, N. K. Bui, m. LeRoux, W. Vollmer, and J. D. Mougous. 2011. Type VI secretion delivers bacteriolytic effectors to target cells. Nature 475.

Russell, A. B., M. LeRoux, K. Hathazi, D. M. Agnello, T. Ishikawa, P. A. Wiggins, S. N. Wai, and J. D. Mougous. 2013. Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. Nature 496:508–12.

Ryan, R. P., S. An, J. H. Allan, Y. McCarthy, and J. M. Dow. 2015. The DSF Family of Cell-Cell Signals: An Expanding Class of Bacterial Virulence Regulators. PLoS Pathog. 11:e1004986.

Ryder, C., M. Byrd, and D. J. Wozniak. 2007. Role of polysaccharides in Pseudomonas aeruginosa biofilm development. Curr. Opin. Microbiol. 10:644–8.

Sampedro, I., R. E. Parales, T. Krell, and J. E. Hill. 2015. Pseudomonas chemotaxis. FEMS Microbiol. Rev. 39:17–46.

Sampedro, I., R. E. Parales, T. Krell, and J. E. Hill. 2014. *Pseudomonas* chemotaxis. FEMS Microbiol. Rev. 20:n/a-n/a.

Sana, T. G., B. Berni, and S. Bleves. 2016. The T6SSs of Pseudomonas aeruginosa Strain PAO1 and Their Effectors: Beyond Bacterial-Cell Targeting. Front. Cell. Infect. Microbiol. 6:61.

Sana, T. G., A. Hachani, I. Bucior, C. Soscia, S. Garvis, E. Termine, J. Engel, A. Filloux, and S. Bleves. 2012. The second type VI secretion system of Pseudomonas aeruginosa strain PAO1 is regulated by quorum sensing and Fur and modulates internalization in epithelial cells. J. Biol. Chem. 287:27095–105.

Sana, T. G., C. Soscia, C. M. Tonglet, S. Garvis, and S. Bleves. 2013. Divergent control of two type VI secretion systems by RpoN in Pseudomonas aeruginosa. PLoS One 8:e76030.

Sauer, K., A. K. Camper, G. D. Ehrlich, J. W. Costerton, and D. G. Davies. 2002. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. J. Bacteriol. 184:1140–1154.

Schaffer, K. 2015. Epidemiology of infection and current guidelines for infection prevention in cystic fibrosis patients. J. Hosp. Infect. 89:309–313.

Schalk, I. J., M. A. Abdallah, and F. Pattus. 2002. Recycling of pyoverdin on the FpvA receptor after ferric pyoverdin uptake and dissociation in Pseudomonas aeruginosa. Biochemistry 41:1663–1671.

Schalk, I. J., and O. Cunrath. 2016. An overview of the biological metal uptake pathways in *P seudomonas aeruginosa*. Environ. Microbiol. 18:3227–3246.

Schuster, M., and P. E. Greenberg. 2006. A network of networks: Quorum-sensing gene regulation in Pseudomonas aeruginosa. Int. J. Med. Microbiol. 296:73–81.

Schwager, S., K. Agnoli, M. Köthe, F. Feldmann, M. Givskov, A. Carlier, and L. Eberl. 2013. Identification of Burkholderia cenocepacia strain H111 virulence factors using nonmammalian infection hosts. Infect. Immun. 81:143–153.

Schwechheimer, C., and M. J. Kuehn. 2015. Outer-membrane vesicles fromGram-negative bacteria: biogenesisand functions. Nat. Rev. Microbiol. 13:605–619.

Sexton, D. J., R. C. Glover, J. E. Loper, and M. Schuster. 2017. Pseudomonas protegens Pf-5 favours self-produced siderophore over free-loading in interspecies competition for iron. Environ. Microbiol. 19:3514–3525.

Silverman, J. M., Y. R. Brunet, E. Cascales, and J. D. Mougous. 2012. Structure and regulation of the type VI secretion system. Annu. Rev. Microbiol. 66:453–72.

Smalley, N. E., D. An, M. R. Parsek, J. R. Chandler, and A. A. Dandekar. 2015. Quorum sensing protects Pseudomonas aeruginosa against cheating by other species in a laboratory co-culture model. J. Bacteriol. 197:3154–3159.

Sorensen, R. U., and J. D. Klinger. 1987. Biological Effects of Pseudomonas aeruginosa Phenazine Pigments. Pp. 113–124 *in* D. G, H. IA, and B. K, eds. Basic Research and Clinical Aspects of Pseudomonas Aeruginosa. International Symposium on Pseudomonas Aeruginosa. Karger Publishers, Tübingen.

Sourjik, V. 2004. Receptor clustering and signal processing in E. coli chemotaxis. Trends Microbiol. 12:569–576.

Starkey, M., J. H. Hickman, L. Ma, N. Zhang, S. de Long, A. Hinz, et al. 2009. Pseudomonas aeruginosa rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. J. Bacteriol. 191:3492–3503.

Stintzi, A., Z. Johnson, M. Stonehouse, U. Ochsner, J.-M. Meyer, M. L. Vasil, and K. Poole. 1999. The pvc gene cluster of Pseudomonas aeruginosa: role in synthesis of the pyoverdine chromophore and regulation by PtxR and PvdS. J. Bacteriol. 181:4118–4124.

Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton. 2002. Biofilms as Complex Differentiated Communities. Annu. Rev. Microbiol. 56:187–209.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, et al. 2000. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 406:959–964.

Stubbendieck, R. M., and P. D. Straight. 2016. Multifaceted interfaces of bacterial competition. J. Bacteriol. 198:2145–2155.

Stubbendieck, R. M., C. Vargas-Bautista, and P. D. Straight. 2016. Bacterial Communities: Interactions to Scale. Front. Microbiol. 7:1234.

Stuer, W., K. E. Jaeger, and U. K. Winkler. 1986. Purification of extracellular lipase from Pseudomonas aeruginosa. J. Bacteriol. 168:1070–4.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, et al. 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A. 102:15545–50.

Sunagawa, S., L. P. Coelho, S. Chaffron, J. R. Kultima, K. Labadie, G. Salazar, et al. 2015. Structure and function of the global ocean microbiome. Science 348:1261359.

Suzuki, Y., S. Kajii, M. Nishiyama, and A. Iguchi. 2013. Susceptibility of Pseudomonas aeruginosa isolates collected from river water in Japan to antipseudomonal agents. Sci. Total Environ. 450–451:148–154.

Tashiro, Y., Y. Yawata, M. Toyofuku, H. Uchiyama, and N. Nomura. 2013. Interspecies interaction between Pseudomonas aeruginosa and other microorganisms. Microbes Environ. 28:13–24.

The Human Microbiome Project. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207–214.

Tielen, P., H. Kuhn, F. Rosenau, K.-E. Jaeger, H.-C. Flemming, and J. Wingender. 2013. Interaction between extracellular lipase LipA and the polysaccharide alginate of Pseudomonas aeruginosa. BMC Microbiol. 13:159.

Tomich, M., P. J. Planet, and D. H. Figurski. 2007. The tad locus: postcards from the widespread colonization island. Nat. Rev. Microbiol. 5:363–375.

Traxler, M. F., J. D. Watrous, T. Alexandrov, P. C. Dorrestein, and R. Kolter. 2013. Interspecies interactions stimulate diversification of the Streptomyces coelicolor secreted metabolome. MBio 4:e00459-13.

Tyrrell, J., N. Whelan, C. Wright, I. Sá-Correia, S. McClean, M. Thomas, and M. Callaghan. 2015. Investigation of the multifaceted iron acquisition strategies of Burkholderia cenocepacia. Biometals 28:367–380.

Vance, R. E., S. Hong, K. Gronert, C. N. Serhan, and J. J. Mekalanos. 2004. The opportunistic pathogen Pseudomonas aeruginosa carries a secretable arachidonate 15-lipoxygenase. Proc. Natl. Acad. Sci. U. S. A. 101:2135–9.

Vasil, M. L. 2006. Pseudomonas aeruginosa Phospholipases and Phospholipids. Pp. 69–97 *in* J.-L. Ramos and R. C. Levesque, eds. Pseudomonas. Springer US, Boston, MA.

Vasil, M. L., and U. A. Ochsner. 1999. MicroReview The response of Pseudomonas aeruginosa to iron : genetics , biochemistry and virulence. 34:399–413.

Vega, N., and J. Gore. 2015. Biofilms : How Structure Emerges from Conflict. Curr. Biol. 25:R800–R802.

Vega, N. M., and J. Gore. 2014. Collective antibiotic resistance: mechanisms and implications. Curr. Opin. Microbiol. 21:28–34.

Venturi, V. 2006. Regulation of quorum sensing in Pseudomonas. FEMS Microbiol. Rev. 30:274–291.

Wade, D. S., M. W. Calfee, E. R. Rocha, E. A. Ling, E. Engstrom, J. P. Coleman, and E. C. Pesci. 2005. Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa. J. Bacteriol. 187:4372–80.

Wagner, V. E., D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski. 2003. Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. 185:2080–2095.

Wang, L.-H., Y. He, Y. Gao, J. E. Wu, Y.-H. Dong, C. He, et al. 2003. A bacterial cell-cell communication signal with cross-kingdom structural analogues. Mol. Microbiol. 51:903–912.

Weaver, V. B., and R. Kolter. 2004. Burkholderia spp. alter Pseudomonas aeruginosa physiology through iron sequestration. J Bacteriol 186:2376–2384.

Weigert, M., A. Ross-Gillespie, A. Leinweber, G. Pessi, S. P. Brown, and R. Kümmerli. 2017. Manipulating virulence factor availability can have complex consequences for infections. Evol. Appl. 10:91–101.

Westhoff, S., G. P. van Wezel, and D. E. Rozen. 2017. Distance-dependent danger responses in bacteria. Curr. Opin. Microbiol. 36:95–101.

Wilderman, P. J., A. I. Vasil, Z. Johnson, M. J. Wilson, H. E. Cunliffe, I. L. Lamont, and M. L. Vasil. 2001. Characterization of an endoprotease (PrpL) encoded by a pvdS-regulated gene in Pseudomonas aeruginosa. Infect. Immun. 69:5385–5394.

Williams, P., and M. Cámara. 2009. Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Curr. Opin. Microbiol. 12:182–191.

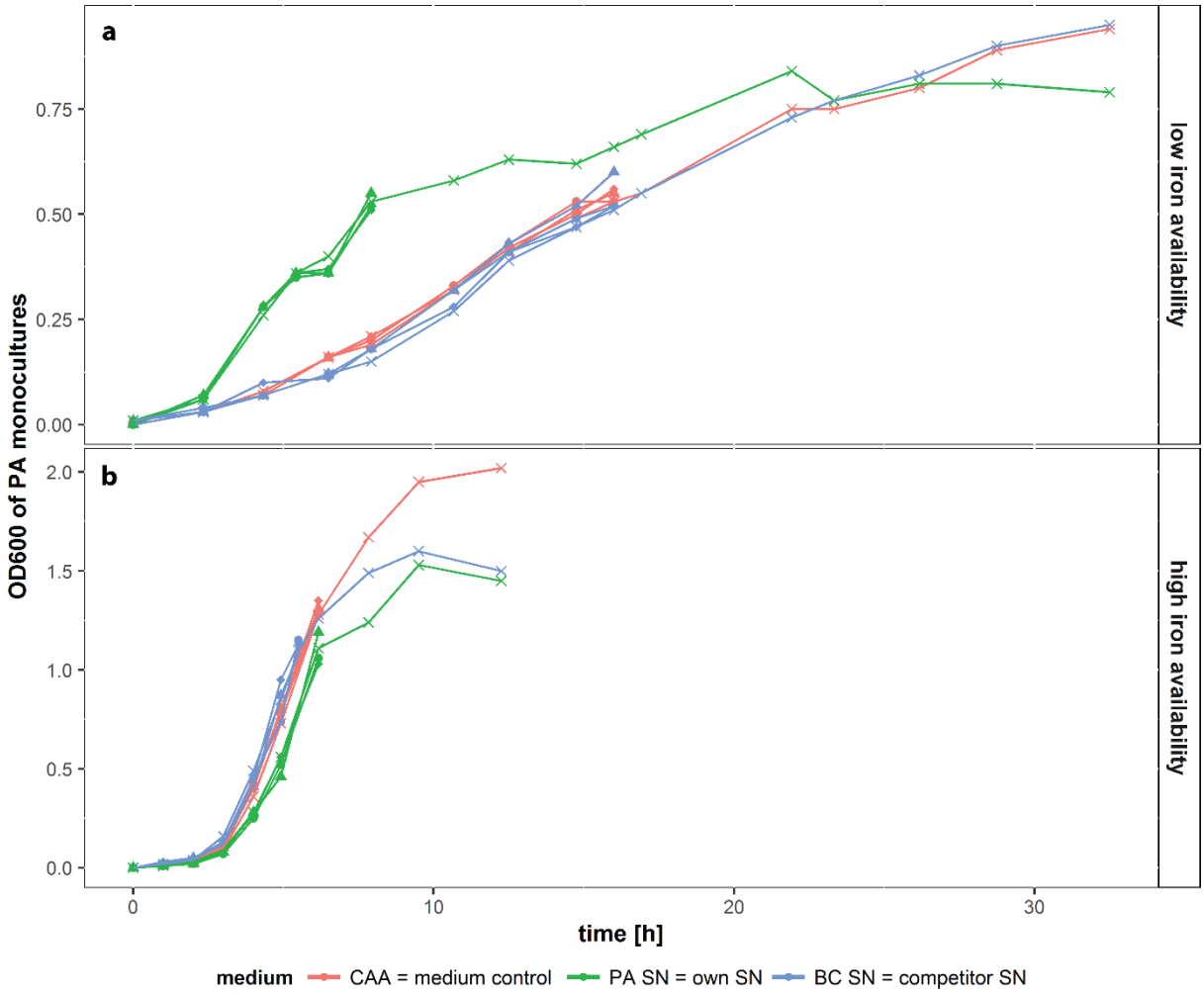
Willsey, G. G., M. J. Wargo, S. Rajamani, J. Allard, L. Lundblad, and G. Allen. 2015. Extracellular Lipase and Protease Production from a Model Drinking Water Bacterial Community Is Functionally Robust to Absence of Individual Members. PLoS One 10:e0143617.

Winsor, G. L., E. J. Griffiths, R. Lo, B. K. Dhillon, J. A. Shay, and F. S. L. Brinkman. 2016. Enhanced annotations and features for comparing thousands of Pseudomonas genomes in the Pseudomonas genome database. Nucleic Acids Res. 44:D646–D653.

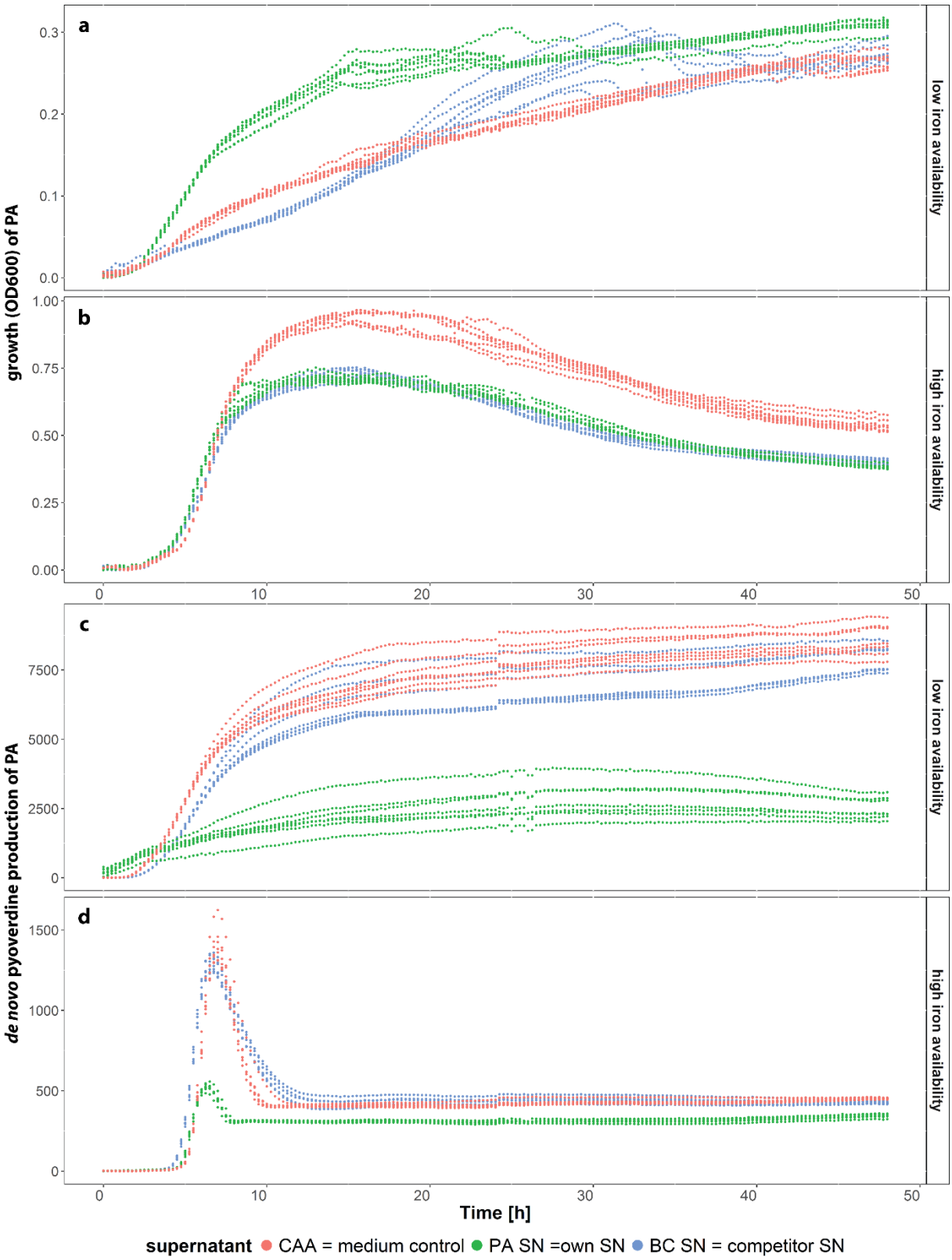
Winzer, K., C. Falconer, N. C. Garber, S. P. Diggle, M. Camara, and P. Williams. 2000. The Pseudomonas aeruginosa lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS. J. Bacteriol. 182:6401–11.

Yamamoto, K., H. Arai, M. Ishii, and Y. Igarashi. 2012. Involvement of flagella-driven motility and pili in Pseudomonas aeruginosa colonization at the air-liquid interface. Microbes Environ. 27:320–3.

5.1. Supporting Information

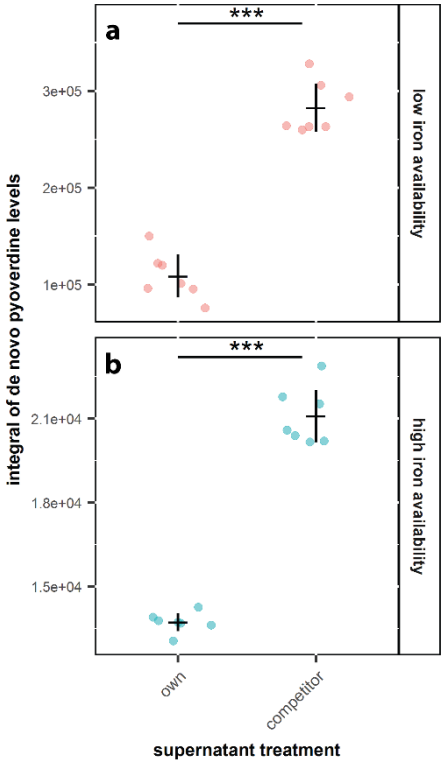


***Supporting Figure 1**: Monitoring growth of P. aeruginosa (PA) to find the right time poiint to harvest for following RNA isolation. PA grew in 100% fresh CAA medium (medium control, red), in 70% fresh CAA medium mixed with 30% of its own (PA) supernatant (green) or mixed with 30% of competitor (B. cenocepacia, BC) supernatant (blue). We used either low iron availability (CAA + 100 µg/ml transferrin) (a) or high iron availability (high iron availability = CAA + 20uM FeCl3) (b). PA was grown in 1 liter Erlenmeyer flasks in 200 ml medium at 37°C and 220 rpm. We followed growth by measuring the OD600 with a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences) until the cultures reached the mid- to late exponential phase, when we harvested the bacteria for following RNA isolation. We grew PA in 3 biological replicates per treatment. Each replicate of the PA and BC supernatant treatment contained a supernatant from an individually grown PA or BC monoculture under the respective iron condition. A fourth replicate was not harvested but was further monitored until the increase of OD600 leveled out, to control for the correct time point of harvesting.*



***Supporting Figure 2**: : Growth and de novo pyoverdine profiles of P. aeruginosa (PA) grown in 100% fresh CAA medium (medium control, red), in 70% fresh CAA medium mixed with 30% of its own (PA) supernatant (green) or mixed with 30% of competitor (B. cenocepacia, BC) supernatant (blue) at low or high iron availability over 48 hours. Under iron limitation, PA growth is stimulated by its own supernatant due to recyclable pyoverdine. The BC supernatant and iron limited CAA medium only do not contain growth stimulating compounds. In contrast, the BC supernatant reduces PA growth compared to the other two treatments during early growth phases (a). Under iron limitation PA and BC supernatants mainly contain spent medium, which reduces PA's overall growth compared to the medium control (b). The recyclable pyoverdine from the PA supernatant leads to a lower de novo pyoverdine synthesis by PA, under iron limitation compared to the other two supernatant treatments*

(c). Under high iron availability the spent culture supernatant from PA suppresses PA's de novo pyoverdine synthesis (d).The pyoverdine profiles over 48 hours demonstrate the temporal dependency of pyoverdine synthesis, which is most active during early growth phases (c,d).Bacteria were cultured under two different iron regiments (low iron availability = CAA + 100 µg/ml transferrin; high iron availability = CAA + 20uM FeCl3). We then measured the OD600 and the autofluorescence of pyoverdine (excitation = 400 nm, emission = 460 nm) over 48 hours of 7 replicates per treatment.



Supporting Figure 3**: De novo pyoverdine production by PA is affected by the iron availability of the medium and the supernatant treatment. Under iron limitation (red), the overall de novo pyoverdine synthesis is reduced by its own supernatant due to recyclable pyoverdine in the supernatant compared to growth in CAA medium mixed with BC (competitor) spent culture supernatant (a). When iron is replete (blue), PA's own supernatant suppresses the de novo pyoverdine synthesis compared to the competitor supernatant, because the supernatant mainly consists of spent medium (b). Diffusible competition cues from the BC supernatant could also cause a higher de novo pyoverdine synthesis as a competitive strategy of PA to capitalize iron before the competitor can do so. In general, a lower iron availability leads to higher de novo pyoverdine synthesis by PA (a), then high iron availability (b). Circles depict individual data points (7 replicates per treatment) and values represent the integral of the global pyoverdine production profile (area under the curve over 48 hours). Black symbols depict means ± 95% confidence intervals. Asterisks indicate significant (P<0.0001) differences between PA grown in fresh medium mixed with its own supernatant or in fresh medium mixed with BC (competitor) supernatant.*

6. Synopsis

Bacteria live in multispecies communities where competition for common resources and space is high, especially when ecological niches of competitors overlap. In the light of this competition, it is unclear how multiple species can co-exist and how they interact with one another. My thesis aimed to address this issue by examining mechanisms of co-existence and competition of bacterial communities under laboratory conditions. I hereby focused on the competition for iron, a limited, yet essential nutrient for almost all living organisms. I used *Pseudomonas aeruginosa* (PA) and *Burkholderia cenocepacia* (BC) as a model system to study interactions between different bacterial species. These species can co-occur in natural habitats e.g. lungs of cystic fibrosis patients (Harrison 2007; Schwab et al. 2014). It was shown in laboratory experiments that the co-existence of these species depends on environmental details, such as high spatial structure (Riedel et al. 2001). Nevertheless, both species can be found regularly in habitats with low spatial structure outside of the lab (Fang et al. 2011; Maravić et al. 2012; Suzuki et al. 2013; Nair et al. 2015). Ecological theory predicts that species coexistence under these conditions is only possible if there are high degrees of within species competition (Chesson 2000). I tested this hypothesis in my first project with a model bacterial community consisting of PA and BC that compete for iron. Both species secrete unique siderophores under iron limitation to scavenge iron from the environment. I manipulated the competition for iron by varying the iron availability in the medium. I further manipulated within species competition by including a PA siderophore non-producer in my experiments. This strain exploits the cooperatively produced siderophore pool of PA and thus saves resources otherwise required for siderophore production. Hence, the siderophore non-producer, also referred to as “cheater”, gains a fitness advantage over the producer and can spread in the population. My experiments identified siderophore-exploitation as a powerful mechanism to increase within-species competition, which in turn fostered co-existence of PA and BC in my model system. Specifically, I found that within species cheating suppressed the fitness of the PA cooperator. This reduced its competitiveness against BC, thus enabling coexistence of BC, yet at low cell densities, with its strong competitor PA. This effect was strongest at low iron availability and low spatial structure, when the fitness of the PA cheater was highest.

In my second project, I addressed the question, whether PA can sense the level of inter-specific competition for iron and adjust its competitive phenotype accordingly. As a model trait, I focused on the production of pyoverdine, PA’s primary siderophore secreted to compete for iron. From an evolutionary point of view, it would be beneficial to express such a metabolically costly phenotype only, when it is needed and confers a fitness advantage over the competitor. This kind of phenotypic plasticity is common in higher organisms. Bacteria are known to express specific phenotypes in competitive environments. PA, for example, responds to antibiotics by increasing biofilm formation,

and produces more pyoverdine in cocultures with other bacterial species (McKenney et al. 1995; Weaver and Kolter 2004; Harrison et al. 2008; Jones et al. 2013; Oliveira et al. 2015). However, it is unclear if the bacterial response is binary (on/off) or if it can be gradually adjusted to different levels of competition. I tackled this problem by comparing pyoverdine production and growth of PA in monocultures and when competing against BC. I changed the level of competition by varying the relative abundance of BC cells and the level of resource competition for iron by manipulating the iron availability in the medium. I found that PA increases early pyoverdine production gradually in response to increasing levels of competition, which results in an early fitness advantage for PA in mixed cultures relative to its monocultures. This fitness advantage manifests in earlier initiation of growth, which is also fine-tuned to match prevailing levels of competition by BC. This plastic phenotype and fitness benefit was most pronounced in iron limited medium, when pyoverdine is essential for iron uptake and thus mediates resource competition between PA and BC the strongest. In contrast, later pyoverdine production and growth rate and yield of PA are reduced in mixed cultures relative to PA monocultures. This fitness reduction correlated with the prevailing level of competition by BC and occurred under all iron treatments. My findings provide conclusive evidence that bacteria are able to fine-tune their phenotype in response to different levels of competition. My results further show, that pyoverdine is an important mediator of the competition between PA and BC. However, my experiments suggest that other factors than pyoverdine are also important for the competition between PA and BC, especially at later competition stages. Therefore, in my third project, I investigated global responses of PA to cues indicating the presence of its competitor BC. I was interested in whether PA can sense diffusible cues of BC and mount specific responses. I therefore grew PA in a mixture of fresh media and spent BC culture supernatant. I used PA grown in its own supernatant and in unconditioned medium as reference treatments. By using RNA sequencing, I measured changes in mRNA levels of genes encoding traits known to be involved in either defense or attack mechanisms. These traits involve biofilm formation, motility, quorum sensing, siderophore-mediated iron uptake, secretion systems, secreted enzymes and other secondary metabolites. I found that diffusible cues from BC lead to substantial gene expression changes in PA, indicating that cues from a distant competitor, and not only direct cell-cell contact, are sufficient to express a competitive phenotype. I found that PA initiates a complex multivariate response to its competitor by regulating several traits simultaneously. Which traits are up- or downregulated depended strongly on the iron availability in the medium, indicating a strong influence of the environment on the competition strategy of PA against BC. Only a few traits were regulated in the same direction under high and low iron availabilities. The synthesis genes for HHQ, a quorum sensing signal precursor that also has antibacterial properties (Wade et al. 2005; Dubern and Diggle 2008; Reen et al. 2011), were upregulated, whereas the genes encoding the AHL based quorum sensing systems of PA, biofilm formation, pyochelin synthesis and the

assembly of the type-3-secretion system were downregulated. When iron availability was limited, the BC supernatant caused an increased expression of hydrogen cyanide and phenazine synthesis genes, as well as the gene encoding the lipase LipA. Under high iron availability, the BC supernatant induces PA to increase the expression of genes encoding a greater number of traits. Here, I found that genes encoding flagella, H1- and H2-type-6-secretion systems and pyocins are increasingly expressed. Also the expression of genes encoding pyoverdine synthesis, as well as the protease PmpA and the lipoxygenase LoxA were upregulated. While several studies examined how PA responds to other species on the level of single traits (McKenney et al. 1995; Weaver and Kolter 2004; Harrison et al. 2008; Oliveira et al. 2015; Bernier et al. 2016), I here conducted a global study of PA's competitive response to inter-specific competition. The number of upregulated traits in response to BC cues could be linked to possible fitness trade-offs I observed in project 2. The various traits which are regulated in response to secreted BC cues could also contribute to PA's dominance over BC in mixed cultures in a wide range of conditions (project 1).

General Discussion

6.1. The importance of studying social interactions in multispecies communities

6.1.1. Effects of social interactions on the course of polymicrobial infections

The human body is colonized by a multitude of microbial species. Some of them are detrimental causing polymicrobial infections (Peters et al. 2012; Quinn et al. 2016). Other bacterial communities are beneficial and form our first line of defense against pathogens (Grice and Segre 2011; Costello et al. 2012; Cresci and Bawden 2015). Despite this high biodiversity of microorganisms in and on the human body, most studies on pathogens only consider the interaction of a single pathogenic species with the host. With this approach we tend to over-simplify and neglect the complex social systems that pathogens are integrated in. In contrast, it would be important to take social interactions between microbes into consideration, since evolutionary changes in pathogens could also be caused by adaptation to the polymicrobial environment (O'Brien and Fothergill 2017). Social interactions between pathogens could also change the course of infections. For example, it has been shown in cell cultures and animal studies that coinfections of *Pseudomonas aeruginosa* (PA) with other bacterial species cause a different immune response than PA mono-infections (Bragonzi et al. 2012; Costello et al. 2014). One example of a polymicrobial disease is the infection of lungs of cystic fibrosis (CF) patients with a multitude of gram-positive and negative bacteria, and also fungi. These infections are a major cause of mortality and morbidity for CF patients. PA is among the most prevalent and dangerous pathogens of CF patients and has therefore been studied extensively (Harrison 2007; Peters et al. 2012; Bhagirath et al. 2016; Quinn et al. 2016). In chronic infections of CF lungs PA becomes less virulent over time by losing or downregulating various virulence factors, e.g. pyoverdine production (Folkesson et al. 2012; Sousa and Pereira 2014; Winstanley et al. 2016). To find reasons for these phenomena, research often focused on the adaptation of PA to its host (Marvig et al. 2015). Over time the tissue of CF lungs becomes damaged and the thick mucus constitutes an anaerobe environment for bacteria, so that the general iron availability and the abundance of other iron sources like heme, increases, so that pyoverdine production in PA might not be essential anymore (Worlitzsch et al. 2002; Alvarez-Ortega and Harwood 2007) (Stites et al. 1999; Cornelis and Dingemans 2013). However, pyoverdine non-producers could also evolve due to social dynamics within the population of PA pathogens and in response to social interactions with other species inside the host (Andersen et al. 2015). If the siderophore non-producers act as cheats on the population of PA cooperators, they could even further facilitate the coexistence of different pathogens in the lungs of CF patients in a similar manner as I have shown in my first project. In response to inter-specific competition PA increases its pyoverdine production above the level required to maintain iron-homeostasis (project 2). The higher amounts of freely available pyoverdine and the additional costs associated with its production could further

accelerate the evolution and invasion of pyoverdine non-producers (Weaver and Kolter 2004; Harrison et al. 2008).

Taken together, the results of my projects demonstrate, how important it is to understand the polymicrobial interactions and their effects on the community as a whole and on the evolution of single community members over time, may it be in environmental communities or in polymicrobial infections.

6.1.2. Social interactions as "by-products" of adaptation to environment and polymicrobial communities

Often it is assumed that competitive traits evolved solely for the purpose of competition. In contrast, it is equally likely that a certain trait has evolved to meet the requirements of a specific environment and that its competitive function is a by-product of its original purpose. Likewise, social interactions between distinct species can have detrimental or beneficial "side effects" on other species or the community. (McNally and Brown 2015).

Competition between bacteria can be a by-product of metabolic activities. For example, metabolic products of fermentation processes in bacteria (e.g. ethanol or lactic acid) can change the environment in a way that other species are negatively affected or even eliminated (Servin 2004; Dashko et al. 2014; Ratzke and Gore 2017; Ratzke et al. 2017). This is a positive effect for the fermenting species, as it reduces the number of competitors for limited resources, but the whole process might not have evolved as a mean to outcompete other species. In contrast, metabolic products can also stabilize biodiversity, when other species can metabolize the end-products of other species' metabolism. This process is called cross-feeding or syntrophy, and is a form of between-species cooperation (Estrela et al. 2012, 2015).

Further, social interactions can arise from an adaptive process to the environment. Pathogenic bacteria damage host tissues, degrade macromolecules and manipulate the host immune system, a process that has been extensively studied in PA infections of CF lungs (Sadikot et al. 2005; Gellatly and Hancock 2013; O'Brien and Fothergill 2017). Thus, pathogens increase nutrient availability and render the environment less hostile for themselves, but simultaneously also for other species, which could facilitate coexistence of multiple species (O'Brien and Fothergill 2017). Bacteria also adapt to the prevailing nutrient availability, which can have consequences for other species as well. In my second project, I found that PA increases pyoverdine production in response to competition by BC. Weaver and Kolter demonstrated that this effect is a reaction to the diminished iron availability due to BC siderophores (Weaver and Kolter 2004). Consequently, the primary goal of increasing pyoverdine

synthesis for PA could be to boost iron uptake from the environment. But, as a side effect, PA also deprives its competitor BC of access to iron, which lowers BC's fitness in the mixed cultures (project 1+2).

Competitive suppression of one microbial species can also be a spill-over from competition between other community members. Toxic compounds that are produced in response to one specific competitor, could inhibit other susceptible not-targeted community members and thus influence the whole community composition. In project 3, I found that PA changes the gene expression upon receiving diffusible cues from BC to induce a multivariate competitive response, including e.g. hydrogen cyanide, pyocyanine synthesis and the type-6-secretion system. Several PA toxins, like the ones mentioned, can have unspecific effects against different bacterial species and the host tissue and thus influence interactions of PA and other species that go beyond the competition of PA and BC.

In my experiments, I investigated the interactions between PA and BC under controlled laboratory conditions. However, natural environments of PA and BC are more complicated. Both species share their natural habitats with polymicrobial communities and species of higher taxa. In addition, they also have to deal with fluctuating environmental conditions. To understand social interactions under these circumstances, using a two-species-community for laboratory experiments in controlled environments is a step in the right direction, but still a simplified model. We would need more studies and advanced technologies to obtain a comprehensive picture of polymicrobial interactions and their role in disease and in maintaining healthy microbiomes.

6.2. Mechanisms of competition in bacteria

6.2.1. Within species competition

Cheating is a common form of competition between isogenic bacterial strains that only differ in the production of one or several public goods. Cheaters gain a fitness advantage over public goods producers by exploiting the cooperative act of public goods production (West et al. 2006). A well-studied system for cheating is the evolution of pyoverdine non-producers in PA capitalizing on the pyoverdine secreted by others for iron uptake (Buckling et al. 2007; Harrison and Buckling 2009; Kümmerli and Brown 2010; Dumas and Kümmerli 2012). But cheaters can evolve for any kind of social trait that can be exploited e.g. quorum sensing or protease secretion (Wilder et al. 2011; Allison et al. 2014; Wang et al. 2015; Mund et al. 2017). I found that within-species competition does not only affect the directly involved strains, but also other species of the community. PA replaced BC in two-way competitions. However, when a siderophore cheater for the dominant species was introduced in the two-species community, within-species competition reduced the between-species competition of PA

with BC. Thereby, the overall fitness of the strong competitor PA was diminished, so that PA and BC could coexist (project 1). Therefore, even social interactions within a small number of community members, can have a large effect on the global polymicrobial community.

6.2.2. Resource competition

Cornforth and Foster (2013) have described two main competition strategies that can be used by bacteria. The first one is indirect exploitative competition, where the competitors increase their resource uptake or accelerate their metabolism in order to deplete resources before their competitor can do so. The second competition strategy is direct interference competition, which I will describe below in section 1.3.3. (Cornforth and Foster 2013).

In my experiments I found that PA and BC engage in resource competition for the limited resource iron (project 1+2). This competition is mediated by their two unique primary siderophores, pyoverdine and ornibactin, respectively. The relative iron affinities of the two siderophores determine which one binds more iron from the environment, and to what extent they are able to remove this essential trace element from natural iron chelators. Thus, the species secreting the more efficient siderophore would win the resource competition and suppress competing species. In my first project I showed that under iron limited conditions PA monocultures grew to a higher yield than BC monocultures, indicating that pyoverdine has a higher iron affinity than ornibactin. I further found that PA can adjust the production of pyoverdine, and therefore its investment in resource competition, to match the prevailing level of competition with BC (project 2). These results demonstrate that siderophores not only mediate within-species competition but can also be used to actively control competition between species.

6.2.3. Interference competition

My results showed that resource competition alone cannot explain PA's dominance over the weaker competitor BC, and highlight that direct interference competition is also involved in displacing BC from mixed cultures (project 1+2). When engaging in direct interference competition, bacteria actively harm each other, e.g. by secreting toxic compounds, in order to replace each other from the shared habitat (Cornforth and Foster 2013). However, the two competition types, resource and interference competition, are not mutually exclusive, but can be used simultaneously or consecutively in order to maximize the fitness of one competitor over the other. This was indeed supported by the results of my third project, where I found that PA changes its gene expression when sensing cues from BC to initiate a potent and multivariate interference competition strategy against BC, involving different secreted toxins and secretion systems. In addition, PA actively increases resource competition via pyoverdine

to suppress its competitor BC (project 2). But, this increased pyoverdine production could as well be interpreted as a means of direct interference competition, because PA actively deprives its competitor of iron, which directly harms BC and lowers its fitness in the competition. Therefore, the distinction between mechanisms of resource versus interference competition is not always clear. Both competition strategies of PA combined, depriving its competitor from essential nutrients and initiating the production of potent toxins, explains PA's strong competitiveness over BC under a variety of conditions (project 1+2).

6.2.4. Competition sensing

In order to initiate resource or interference competition, bacteria need to sense the presence of within and between species competitors. Cornforth and Foster have reviewed the mechanisms that could contribute to competition sensing (introduction 1.1.4.). In short, competition sensing can be mediated by sensing ecological stress in the form of environmental changes (e.g. resource limitation), or by directly sensing competitors through their secreted compounds (e.g. QS molecules, toxins) or through cell-cell contact (Cornforth and Foster 2013). My second and third project tested aspects of the competition sensing hypothesis and revealed that PA has indeed evolved mechanisms to sense different strengths of competition (project 2). PA gradually increases its pyoverdine production to match the prevailing level of competition by BC, which leads to gradual adjustments of PA's fitness in the direct competition with BC.

The question is whether bacteria depend on direct cell-cell contact to initiate a specific response, or whether diffusible cues that reach bacteria before the actual competitor does, are sufficient. Westhoff et al. summarized studies that showed bacterial responses to cues that confer the presence of competitors in different distances. Their categories range from very close (transmitted through cell-cell contact), over intermediate (diffusible cues) to long distances (volatile cues) (Westhoff et al. 2017). An indication that PA and BC can influence each other over larger distances has been found by Schwab et al. 2014. PA and BC co-infect the spatially highly organized lungs of CF patients. This environment provides micro-compartments, which should in theory lower competition between species (Kerr et al. 2002; Kim et al. 2008; Vos et al. 2013). However, the presence of BC still leads to a changed phenotype of PA, even without having direct cell-cell contact. In mixed infections, PA formed less biofilm structures than in PA mono-infections, but could rather be found as single cells in CF lung tissue (Schwab et al. 2014). For these phenotypic changes to occur, the high spatial structure in CF lungs apparently allows smaller secreted compounds to travel from one compartment to another, but it limits the distribution of bacterial cells themselves.

My experiments confirmed that competition sensing over larger distances via diffusible cues plays an important role in the competition between PA and BC. I showed that diffusible cues from BC cause a profound change in PA's gene regulation. PA initiates a diverse response to BC derived cues, which also includes contact dependent inhibition strategies (e.g. type-6-secretion system) (project 3). This could indicate that PA prepares for a yet to come direct encounter with BC. Even though my third project generated a global picture of the initial response of PA to diffusible BC cues, some questions are still unanswered. First, the actual nature of the BC derived cues, which trigger the transcriptional changes in PA, are still obscure. It is not clear if PA responds directly to single cues or to a combination of different cues, or if the secreted compounds from BC cause environmental changes that PA responds to. In my experiments, I used BC spent culture supernatants that contain a multitude of BC derived compounds, which allows one or multiple of the above mechanisms to operate. Second, with RNA sequencing I examined the transcriptional changes and with that the initial response of PA to competitive cues derived from BC. However, which traits are finally used by PA to compete against BC depends also on post-transcriptional and post-translational regulatory mechanisms. Therefore, to fully understand the competitive response of PA against BC, one would also need to analyze the proteome and the metabolome in addition to the transcriptome.

6.3. The role of the environment on bacterial interaction patterns

I found that social interactions influence a multispecies community at the global level by changing species composition (project 1), but also affect gene expression (project 3) and phenotypes of individual community members (project 2). These effects were profoundly altered by the environment in which the bacterial communities were cultured. For example, I found that the spatial structure and the availability of iron fundamentally influenced the outcome and the type of inter-specific competition between PA and BC (project 1). The iron availability also strongly changed PA's gene regulation in response to diffusible cues from its competitor BC (project 3), which shows that responses to competition between bacteria vary in different environmental conditions. While I kept other environmental factors constant in my experiments, it is important to note that in natural habitats they likely vary both at the spatial and temporal scale. This means that bacteria not only need to be able to respond to competitors but also cope with fluctuating environmental conditions. I will now briefly elaborate how such fluctuations could affect the two main environmental variables manipulated in my studies: iron availability and spatial structure.

Bioavailability of iron is known to vary in response to pH and oxygen saturation. From alkaline to acidic environments the oxidative state of iron shifts from the insoluble ferric to the readily soluble ferrous

iron. Therefore, under low pH-values iron is assumed to be more easily accessible for bacteria (Crichton 2001). Since bacteria can change the pH of their environment by secreting primary and secondary metabolites (Hunter and Beveridge 2005; Ratzke and Gore 2017; Ratzke et al. 2017), they could directly influence the iron availability in the surroundings making it more or less available for themselves, but also for other community members including competitors. Another abiotic factor that influences iron bioavailability is the oxygen saturation of the environment. Under low oxygen content the ratio of oxidized (ferric) to reduced (ferrous) iron is shifted towards the easily bioavailable ferrous iron (Crichton 2001). Oxygen saturation in nature can shift across relatively short distances, as regularly observed in soil, but also in lungs of immunocompromised cystic fibrosis patients (Zehnder and Stumm 1988; Lüdemann et al. 2000; Worlitzsch et al. 2002). This could lead to a locally higher bioavailability of iron, which in turn changes the parameters for resource competition.

The second environmental factor I manipulated in my experiments was spatial structure. I found that it had a profound influence on the coexistence of PA and BC (project 1). The two bacterial species can be found in differently structured habitats (e.g. soil, CF lung tissue, aquatic and marine habitats), of which the spatial organization can be further influenced by external parameters like weather (e.g. rain), water currents, or clearance of mucus from CF lungs. Therefore, it is important to understand how spatial structure affects diffusion and dispersal of larger molecules and bacteria themselves. A higher spatial structure restricts diffusion of secreted compounds and dispersal of bacteria. Thus, higher spatial structure can stabilize cooperation by keeping secreted public goods close to the producers, and restricting cooperating clone mates from dispersing (project 1) (Kümmerli et al. 2009b; Weigert and Kümmerli 2017). Furthermore, spatial structure strongly influences inter-specific competition by providing refuge for weaker competitors (Kerr et al. 2002; Kim et al. 2008; Vos et al. 2013). Also for the coexistence of PA and BC, spatial structure plays an important role. Some studies demonstrate that it is possible to establish PA and BC mixed biofilms, which are naturally well structured (Riedel et al. 2001). However, planktonic co-cultures have proved difficult to establish (project 1) (Bragonzi et al. 2012; Costello et al. 2014). Nevertheless, it is possible for bacteria to influence each other when they are spatially separated by high spatial structure, and not only when they have direct cell-cell contact with each other (Schwab et al. 2014). Under high spatial structure, the relatively large bacteria are locally restricted, whereas secreted macromolecules and smaller compounds can still diffuse more freely (Cornforth and Foster 2013; Westhoff et al. 2017). I showed in my third project, that diffusible cues from BC cause large changes in the transcriptome of PA. This could explain the phenomenon in CF patients, that PA shows an altered phenotype in co-infections with BC compared to PA mono-infections, although the two species do not form mixed biofilms (Schwab et al. 2014). Still, PA and BC are able to coexist in the CF lung, whereas the spatially structured

medium in my experiments did not save BC from being outcompeted by PA (project 1). The medium I used in my experiments did not completely separate PA and BC from each other in contrast to host tissue (Schwab et al. 2014). The lung tissue however probably only allows smaller signal molecules to travel between the species, which might not allow PA to completely displace BC from the lung.

Taken together, my experiments showed that the environment determines how bacteria interact with each other on the species and on the community level. The environment can directly manipulate the spatial distribution of bacteria and secreted compounds, and it can manipulate nutrient availability, which mediates competition for common resources. Thus, the environment facilitates bacterial cooperation or the evolution of social cheaters, and it determines if species outcompete, only suppress or co-exist with each other.

6.4. Why should we care about social interactions between bacteria?

Bacteria are ubiquitous in nature and profoundly affect life of higher organisms. They colonize them with beneficial and detrimental effects on their health alike. First, bacteria form multispecies communities as beneficial commensals e.g. on skin or inside the gut, helping their hosts to ward of pathogens and to digest food, respectively (Grice and Segre 2011; Fredricks 2013; Cresci and Bawden 2015). Second, bacteria colonize their hosts in multispecies infections causing severe diseases e.g. lung infections in CF patients (Harrison 2007; Leggett et al. 2014; O'Brien and Fothergill 2017). In addition, bacteria are indispensable for us humans in terms of biotechnological applications. As bacteria naturally live in multispecies communities, it is important to understand the implications of interactions between the community members on each other and their overall effect on the community functioning. *Pseudomonads* are a good example of ubiquitous bacteria that have different implications for humans. The group of *Pseudomonads* includes critical pathogens for immunocompromised patients like *Pseudomonas aeruginosa* (PA) that can also infect plants, other animals and even invertebrates. Since PA is highly resistant to many antibiotics, it is very hard to treat (Novik et al. 2015). However, *Pseudomonads* also exhibit phenotypes that are beneficial for humans, e.g. in bioremediation of polluted soil. Since *Pseudomonads*, including PA, can use a wide range of carbon sources as nutrients they are used for bioremediation to detoxify soil contaminated with organic compounds (Silby et al. 2011). The siderophores secreted by *Pseudomonads*, which do not only bind iron with high affinity but also other metals, could potentially be used for heavy metal bioremediation (O'Brien et al. 2014).

Furthermore, PA is a source of low-molecular-weight compounds like enzymes and rhamnolipids (biosurfactants), that can be used in cosmetics, food or agricultural industry (Jaeger et al. 1996;

Grbavcic et al. 2011; Novik et al. 2015; Sinumvayo and Ishimwe 2015). Moreover, *Pseudomonads*, including PA, can also be useful as plant protectants and plant growth promoters in agriculture (Preston 2004; Hardoim et al. 2015). Hariprasad et al found PA strains in the rhizosphere of tomato plants protecting the plants from pathogens (Hariprasad et al. 2014).

I have shown in my projects that the fitness of PA is highly influenced by social interactions within and between species. It is therefore conceivable that we need to understand how interactions between PA and other bacteria impact the phenotype of PA that we wish to make use of, both in the case where we want them to be expressed for beneficial applications, or suppressed in infectious diseases.

6.4.1. Effect of bacterial interactions on biotechnological applications

In biotechnology, it is important to maximize the amount of a target product that will be isolated from a bacterial culture. Low-molecular-weight compounds that are used for biotechnological applications are often secreted products that can function as public goods, like rhamnolipids and secreted enzymes from PA (Jaeger et al. 1996; Grbavcic et al. 2011; Novik et al. 2015; Sinumvayo and Ishimwe 2015). Therefore, it is important to consider the evolution of non-producers, which could lower the fitness of the producing strain, and overall could reduce the total amount of the target product. Increasing the spatial structure of the medium could be one possibility to stabilize the cooperative production (compare project 1) (Kümmerli et al. 2009b; Weigert and Kümmerli 2017). Further, social interactions could potentially be used to increase the harvested amount of the product. If the target compound is increasingly produced in mixed species cultures, like siderophores, hydrolytic enzymes or extracellular matrix molecules for biofilm formation of PA, adding signals from the competing species to the target bacterial culture could increase the amounts of the final product (compare project 2+3) (Weaver and Kolter 2004; Harrison et al. 2008; Oliveira et al. 2015).

6.4.2. Effect of bacterial interactions on ecosystem functions

Competitive interactions can have strong effects on the composition of multispecies communities, and hence also on the ecological function of this community. Usually, established communities are at an equilibrium based on interactions of bacteria with the environment and with other species. Thereby, the community can fulfill a certain function to the ecosystem that is usually defined following human standards (Alcamo et al. 2003). The predominant opinion is, that a more diverse community is better in fulfilling its ecosystem function (Bell et al. 2005; Hooper et al. 2005). Examples are plant protection, plant growth promotion or soil bioremediation, which are used by agricultural and environmental industries. Furthermore, complex communities of commensal microorganisms are crucial for human

health, as they defend us from pathogens and modulate digestion. Disturbance of the community composition can lead to lower overall biomass yields, and thus disrupt the ecosystem functions. Antibiotic treatments, for example, have adverse effects on the human microbiome, which can lead to digestive problems or an increased susceptibility to other infections (Keeney et al. 2014; Jandhyala et al. 2015; Ianiro et al. 2016).

It is important to note that a higher biodiversity in bacterial communities, e.g. by artificially increasing the abundance of a target bacterial species, does not necessarily correspond to increased ecosystem functioning. For example, for bioremediation it seems to be beneficial to inoculate the polluted soil with bacterial species that can specifically degrade or bind the prevailing pollutant. However, in practice this procedure is very hard to control due to antagonistic interactions of the inoculated species with the native microbial community (Azubuike et al. 2016). The same relationship has been found by Becker et al in a *Pseudomonas fluorescence* community in the rhizosphere, where a higher genotypic richness lead to more antagonistic interactions between different strains, which resulted in less plant growth protection (Becker et al. 2012). Therefore, any interference in natural bacterial communities in order to improve a specific function should be done carefully and monitored closely to avoid excessive antagonism between species, which could otherwise completely interrupt ecosystem functioning.

6.4.3. Effect of bacterial interactions for medical applications

Bacterial communities in the form of microbiomes and polymicrobial infections are important for our health in both beneficial and harmful aspects (Peters et al. 2012). The social interactions between and within the different polymicrobial communities, and also the interactions with the host, can determine the course of infections, and could also change the course of drug treatments (Smith et al. 2003; Eswarappa et al. 2012; Leggett et al. 2014; Quinn et al. 2016). Hence, we need to better understand social interactions between different species in these communities.

The rise of multidrug resistant pathogens poses a large threat to human health care. This is a very urgent problem, as new antibiotics are currently rarely released on the market, while multidrug resistant bacteria are spreading worldwide. The World Health Organization published a list of the most important resistant bacteria at a global level to channel research and development of new antibiotics. This list classifies PA among the highest priority (World Health Organization 2017). Recent research suggests that understanding social interactions between bacteria can lead to the discovery of new drugs or drug targets. One possibility is to target cooperative strategies of bacteria, like quorum sensing and siderophore secretion. To target quorum sensing systems could be successful, as it

regulates many virulence factors in bacteria. Quorum quenchers could be applied that target quorum sensing signals and disrupt communication between bacteria (Hentzer et al. 2002, 2003; Chu et al. 2013; Wagner et al. 2016). In addition, it could also be possible to use cooperatively secreted substances as transporters for drugs inside the bacterial cells. This approach has been tested with antibiotics that were connected to siderophores. Since most siderophores are monopolized by a single species due to specific receptors, this approach could specifically target single species (Saha et al. 2013; Wagner et al. 2016; Schalk and Mislin 2017). Another promising method is to disturb the cooperative iron uptake through siderophores by blocking these molecules with another metal, that cannot be reduced and thus interrupts siderophore recycling (Kaneko et al. 2007; Ross-Gillespie et al. 2014; Weigert et al. 2017). Therefore, quorum and siderophore quenching do not kill bacteria, but reduce the production of virulence factors, which could negatively affect the fitness of the pathogens inside the host, and provide the host immune system the opportunity to remove the pathogens itself. However, in some diseases, like CF, bacteria stop producing siderophores over time (De Vos et al. 2001; Marvig et al. 2014; Nguyen et al. 2014). This could limit the effectiveness of siderophore-drug-complexes and siderophore quenching. Therefore, it is essential to understand the effect that inter-specific competition has on the production of siderophores, to reliably predict the treatment outcome.

Finally, the mechanisms of direct bacterial competition via secreted enzymes and secondary metabolites could be the basis of new drug discoveries. Currently, the search for new bioactive compounds is often conducted in single species that are investigated under standard laboratory conditions. But, bacteria often do not express their full metabolic potential in monocultures and only switch to a competitive phenotype, secreting bioactive compounds, when receiving cues from competitors (project 2+3). Therefore, investigating co-cultures of naturally co-occurring species could trigger the synthesis of compounds, that could potentially become new remedies against multidrug resistant pathogens (Marmann et al. 2014).

Literature

The following list of publications includes citations for the general introduction and discussion. Publications cited in the three separate chapters can be found at the end of their respective chapter.

Agnoli, K., C. A. Lowe, K. L. Farmer, S. I. Husnain, and M. S. Thomas. 2006a. The ornibactin biosynthesis and transport genes of Burkholderia cenocepacia are regulated by an extracytoplasmic function factor which is a part of the fur regulon. J. Bacteriol. 188:3631–3644.

Agnoli, K., C. A. Lowe, K. L. Farmer, S. I. Husnain, and M. S. Thomas. 2006b. The ornibactin biosynthesis and transport genes of Burkholderia cenocepacia are regulated by an extracytoplasmic function sigma factor which is a part of the Fur regulon. J. Bacteriol. 188:3631–44.

Agrawal, A. A. 2001. Phenotypic plasticity in the interactions and evolution of species. Science 294:321–326.

Ahmed, E., and S. J. M. Holmström. 2014. Siderophores in environmental research: roles and applications. Microb. Biotechnol. 7:196.

Alcamo, J., E. M. Bennett, and Millennium Ecosystem Assessment (Program). 2003. Ecosystems and human well-being : a framework for assessment. Island Press.

Allen, B., J. Gore, and M. A. Nowak. 2013. Spatial dilemmas of diffusible public goods. Elife 2:e01169.

Allison, S. D., L. Lu, A. G. Kent, and A. C. Martiny. 2014. Extracellular enzyme production and cheating in Pseudomonas fluorescens depend on diffusion rates. Front. Microbiol. 5:169.

Alvarez-Ortega, C., and C. S. Harwood. 2007. Response of Pseudomonas aeruginosa to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. Mol. Microbiol. 65:153–165.

Andersen, S. B., R. L. Marvig, S. Molin, H. K. Johansen, and A. S. Griffin. 2015. Long-term social dynamics drive loss of function in pathogenic bacteria. Proc. Natl. Acad. Sci. U. S. A. 112:10756–10761.

Asfahl, K. L., and M. Schuster. 2017. Social interactions in bacterial cell–cell signaling. FEMS Microbiol. Rev. 41:92–107.

Aujoulat, F., F. Roger, A. Bourdier, A. Lotthé, B. Lamy, H. Marchandin, and E. Jumas-Bilak. 2012. From environment to man: genome evolution and adaptation of human opportunistic bacterial pathogens. Genes. 3:191–232.

Azubuike, C. C., C. B. Chikere, and G. C. Okpokwasili. 2016. Bioremediation techniques-classification based on site of application: principles, advantages, limitations and prospects. World J. Microbiol. Biotechnol. 32:180.

Baron, S. S., G. Terranova, and J. J. Rowe. 1989. Molecular mechanism of the antimicrobial action of pyocyanin. Curr. Microbiol. 18:223–230.

Becker, J., N. Eisenhauer, S. Scheu, and A. Jousset. 2012. Increasing antagonistic interactions cause bacterial communities to collapse at high diversity. Ecol. Lett. 15:468–474.

Bell, T., J. A. Newman, B. W. Silverman, S. L. Turner, and A. K. Lilley. 2005. The contribution of species richness and composition to bacterial services. Nature 436:1157–1160.

Beneduzi, A., A. Ambrosini, and L. M. P. Passaglia. 2012. Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. Genet. Mol. Biol. 35:1044–51.

Bernier, S. P., M. L. Workentine, X. Li, N. A. Magarvey, G. A. O'Toole, and M. G. Surette. 2016. Cyanide toxicity to Burkholderia cenocepacia is modulated by polymicrobial communities and environmental factors. Front. Microbiol. 7:725.

Bhagirath, A. Y., Y. Li, D. Somayajula, M. Dadashi, S. Badr, and K. Duan. 2016. Cystic fibrosis lung environment and Pseudomonas aeruginosa infection. BMC Pulm. Med. 16:174.

Boege, K. 2010. Induced responses to competition on multi-trait phenotypic plasticity. Ecology 91:2628–2637.

Bragonzi, A., I. Farulla, M. Paroni, K. B. Twomey, L. Pirone, N. I. Lore, et al. 2012. Modelling co-infection of the cystic fibrosis lung by Pseudomonas aeruginosa and Burkholderia cenocepacia reveals influences on biofilm formation and host response. PLoS One 7:e52330.

Brandel, J., N. Humbert, M. Elhabiri, I. J. Schalk, G. L. A. Mislin, and A.-M. Albrecht-Gary. 2012. Pyochelin, a siderophore of Pseudomonas aeruginosa: physicochemical characterization of the iron(III), copper(II) and zinc(II) complexes. Dalt. Trans. 41:2820–2834.

Bruger, E., and C. Waters. 2015. Sharing the sandbox: Evolutionary mechanisms that maintain bacterial cooperation. F1000Research 4:1504.

Buckling, A., F. Harrison, M. Vos, M. A. Brockhurst, A. Gardner, S. A. West, and A. S. Griffin. 2007. Siderophore-mediated cooperation and virulence in Pseudomonas aeruginosa. Fems Microbiol. Ecol. 62:135–141.

Butaitė, E., M. Baumgartner, S. Wyder, and R. Kümmerli. 2017. Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater Pseudomonas communities. Nat. Commun. 8:414.

Buyer, J. S., and J. Leong. 1986. Iron transport-mediated antagonism between plant growth-promoting and plant-deleterious Pseudomonas strains. J. Biol. Chem. 261:791–794.

Cao, H., R. L. Baldini, and L. G. Rahme. 2001. Common mechanisms for pathogens of plants and animals. Annu. Rev. Phytopathol. 39:259–284.

Carlier, A., K. Agnoli, G. Pessi, A. Suppiger, C. Jenul, N. Schmid, B. Tümmler, M. Pinto-Carbo, and L. Eberl. 2014. Genome Sequence of Burkholderia cenocepacia H111, a Cystic Fibrosis Airway Isolate. Genome Announc. 2:e00298-14.

Chesson, P. 2000. Mechanisms of maintenance of species diversity. Annu. Rev. Ecol. Evol. Syst. 31:343–366.

Chu, B. C., A. Garcia-Herrero, T. H. Johanson, K. D. Krewulak, C. K. Lau, R. S. Peacock, Z. Slavinskaya, and H. J. Vogel. 2010. Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view. Biometals 23:601–611.

Chu, Y.-Y., M. Nega, M. Wölfle, L. Plener, S. Grond, K. Jung, and F. Götz. 2013. A New Class of Quorum Quenching Molecules from Staphylococcus Species Affects Communication and Growth of Gram-Negative Bacteria. PLoS Pathog. 9:e1003654.

Coenye, T., and P. Vandamme. 2003. Diversity and significance of Burkholderia species occupying diverse ecological niches. Environ. Microbiol. 5:719–729.

Cornelis, P. 2010. Iron uptake and metabolism in pseudomonads. Appl. Microbiol. Biotechnol. 86:1637–1645.

Cornelis, P., and J. Dingemans. 2013. Pseudomonas aeruginosa adapts its iron uptake strategies in function of the type of infections. Front. Cell. Infect. Microbiol. 3:1–7.

Cornelis, P., S. Matthijs, and L. van Oeffelen. 2009. Iron uptake regulation in Pseudomonas aeruginosa. Biometals 22:15–22.

Cornforth, D. M., and K. R. Foster. 2013. Competition sensing: the social side of bacterial stress responses. Nat. Rev. Microbiol. 11:285–293.

Costello, A., F. J. Reen, F. O'Gara, M. Callaghan, and S. McClean. 2014. Inhibition of co-colonizing cystic fibrosis-associated pathogens by Pseudomonas aeruginosa and Burkholderia multivorans. Microbiology 160:1474–1487.

Costello, E. K., K. Stagaman, L. Dethlefsen, B. J. M. Bohannan, and D. A. Relman. 2012. The application of ecological theory toward an understanding of the human microbiome. Science 336:1255–62.

Cresci, G. A., and E. Bawden. 2015. Gut Microbiome: What We Do and Don't Know. Nutr. Clin. Pract. 30:734–46.

Crichton, R. R. 2001. Inorganic biochemistry of iron metabolism : from molecular mechanisms to clinical consequences. 2nd ed. John Wiley & Sons, Ltd, Chichester, West Sussex, England.

D'Onofrio, A., J. M. Crawford, E. J. Stewart, K. Witt, E. Gavrish, S. Epstein, J. Clardy, and K. Lewis. 2010. Siderophores from neighboring organisms promote the growth of uncultured bacteria. Chem. Biol. 17:254–264.

Dashko, S., N. Zhou, C. Compagno, and J. Piškur. 2014. Why, when, and how did yeast evolve alcoholic fermentation? FEMS Yeast Res. 14:826–32.

De Vos, D., M. De Chial, C. Cochez, S. Jansen, B. Tümmler, J.-M. Meyer, and P. Cornelis. 2001. Study of pyoverdin type and production by Pseudomonas aeruginosa isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine-negative mutations. Arch. Microbiol. 175:384–388.

Drevinek, P., and E. Mahenthiralingam. 2010. Burkholderia cenocepacia in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin. Microbiol. Infect. 16:821–830.

Dubern, J.-F., and S. P. Diggle. 2008. Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species. Mol. Biosyst. 4:822–888.

Dumas, Z., and R. Kümmerli. 2012. Cost of cooperation rules selection for cheats in bacterial metapopulations. J. Evol. Biol. 25:473–484.

Dumas, Z., A. Ross-Gillespie, and R. Kümmerli. 2013. Switching between apparently redundant iron-uptake mechanisms benefits bacteria in changeable environments. Proc. R. Soc. B 280:20131055.

Dunny, G. M., T. J. Brickman, and M. Dworkin. 2008. Multicellular behavior in bacteria: Communication, cooperation, competition and cheating. BioEssays 30:296–298.

Eberl, L., and P. Vandamme. 2016. Members of the genus Burkholderia: good and bad guys. F1000Research 5:1007.

Estrela, S., C. H. Trisos, and S. P. Brown. 2012. From metabolism to ecology: cross-feeding interactions shape the balance between polymicrobial conflict and mutualism. Am. Nat. 180:566–76.

Estrela, S., M. Whiteley, and S. P. Brown. 2015. The demographic determinants of human microbiome health. Trends Microbiol. 23:134–141.

Eswarappa, S. M., S. Estrela, and S. P. Brown. 2012. Within-host dynamics of multi-species infections: facilitation, competition and virulence. PLoS One 7:e38730.

Fang, Y., G. Xie, M. Lou, B. Li, and I. Muhammad. 2011. Diversity analysis of Burkholderia cepacia complex in the water bodies of West Lake, Hangzhou, China. J. Microbiol. 49:309–314.

Fazli, M., H. Almblad, M. L. Rybtke, M. Givskov, L. Eberl, and T. Tolker-Nielsen. 2014. Regulation of biofilm formation in Pseudomonas and Burkholderia species. Environ. Microbiol. 16:1961–1981.

Filkins, L. M., J. A. Graber, D. G. Olson, E. L. Dolben, L. R. Lynd, S. Bhuju, and G. A. O'Toole. 2015. Co-culture of Staphylococcus aureus with Pseudomonas aeruginosa drives S. aureus towards fermentative metabolism and reduced viability in a cystic fibrosis model. J. Bacteriol. 197:2252–2264.

Flemming, H.-C., J. Wingender, U. Szewzyk, P. Steinberg, S. A. Rice, and S. Kjelleberg. 2016. Biofilms: an emergent form of bacterial life. Nat. Rev. Microbiol. 14:563–575.

Folkesson, A., L. Jelsbak, L. Yang, H. K. Johansen, O. Ciofu, N. Hoiby, and S. Molin. 2012. Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol 10:841–851.

Fox, B. G., Fox, and B. G. 2015. Iron Cofactors: Nonhaem. Pp. 1–8 *in* eLS. John Wiley & Sons, Ltd, Chichester, UK.

Fredricks, D. N. (ed). 2013. The human microbiota : how microbial communities affect health and disease. Wiley-Blackwell.

Galet, J., A. Deveau, L. Hôtel, P. Frey-Klett, P. Leblond, and B. Aigle. 2015. Pseudomonas fluorescens pirates both ferrioxamine and ferricoelichelin siderophores from Streptomyces ambofaciens. Appl. Environ. Microbiol. 81:3132–3141.

Gellatly, S. L., and R. E. W. Hancock. 2013. *Pseudomonas aeruginosa* : new insights into pathogenesis and host defenses. Pathog. Dis. 67:159–173.

Girard, G., and G. V Bloemberg. 2008. Central role of quorum sensing in regulating the production of pathogenicity factors in *Pseudomonas aeruginosa*. Future Microbiol. 3:97–106.

Goo, E., J. H. An, Y. Kang, and I. Hwang. 2015. Control of bacterial metabolism by quorum sensing. Trends Microbiol. 23:1–10.

Granato, E. T., F. Harrison, R. Kümmerli, and A. Ross-Gillespie. 2016. Do Bacterial Virulence Factors Always Increase Virulence? A Meta-Analysis of Pyoverdine Production in Pseudomonas aeruginosa As a Test Case. Front. Microbiol. 7:1952.

Grbavcic, S., D. Bezbradica, L. Izrael-Zivkovic, N. Avramovic, N. Milosavic, I. Karadzic, and Z. Knezevic-Jugovic. 2011. Production of lipase and protease from an indigenous Pseudomonas aeruginosa strain and their evaluation as detergent additives: Compatibility study with detergent ingredients and washing performance. Bioresour. Technol. 102:11226–11233.

Grice, E. A., and J. A. Segre. 2011. The skin microbiome. Nat. Rev. Microbiol. 9:244–53.

Griffin, A. S., S. A. West, and A. Buckling. 2004. Cooperation and competition in pathogenic bacteria. Nature 430:1024–1027.

Haas, D., and G. Défago. 2005. Biological control of soil-borne pathogens by fluoresecent Pseudomonads. Nat. Rev. Microbiol. 3:307–319.

Hamilton, W. D. 1963. The evolution of altruistic behavior. Am. Nat. 97:354–356.

Hamilton, W. D. 1964. The genetical evolution of social behaviour. J. Theor. Biol. 7:1–52.

Hardoim, P. R., L. S. van Overbeek, G. Berg, A. M. Pirttilä, S. Compant, A. Campisano, M. Döring, and A. Sessitsch. 2015. The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiol. Mol. Biol. Rev. 79:293–320.

Hariprasad, P., S. Chandrashekar, S. B. Singh, and S. R. Niranjana. 2014. Mechanisms of plant growth promotion and disease suppression by *Pseudomonas aeruginosa* strain 2apa. J. Basic Microbiol. 54:792–801.

Harrington, J. M., O. W. Duckworth, and K. Haselwandter. 2015. The fate of siderophores: antagonistic environmental interactions in exudate-mediated micronutrient uptake. BioMetals 28:461–472.

Harrison, F. 2007. Microbial ecology of the cystic fibrosis lung. Microbiology 153:917–923.

Harrison, F., L. E. Browning, M. Vos, and A. Buckling. 2006. Cooperation and virulence in acute Pseudomonas aeruginosa infections. BMC Biol. 4:21.

Harrison, F., and A. Buckling. 2009. Cooperative production of siderophores by Pseudomonas aeruginosa. Front. Biosci. 14:4113–4126.

Harrison, F., J. Paul, R. C. Massey, and A. Buckling. 2008. Interspecific competition and siderophore-mediated cooperation in Pseudomonas aeruginosa. ISME J. 2:49–55.

Hawlena, H., F. Bashey, H. Mendes-Soares, and C. M. Lively. 2010. Spiteful interactions in a natural population of the bacterium Xenorhabdus bovienii. Am. Nat. 175:374–381.

Hayes, C. S., S. Koskiniemi, Z. C. Ruhe, S. J. Poole, and D. A. Low. 2014. Mechanisms and biological roles of contact-dependent growth inhibition systems. Cold Spring Harb. Perspect. Med. 4:a010025.

Hentzer, M., K. Riedel, T. B. Rasmussen, A. Heydorn, J. B. Andersen, M. R. Parsek, et al. 2002. Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound. Microbiology 148:87–102.

Hentzer, M., H. Wu, J. B. Andersen, K. Riedel, T. B. Rasmussen, N. Bagge, et al. 2003. Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. EMBO J. 22:3803–15.

Hibbing, M. E., C. Fuqua, M. R. Parsek, and S. B. Peterson. 2010. Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8:15–22.

Ho, B. T., T. G. Dong, and J. J. Mekalanos. 2014. A View to a Kill : The Bacterial Type VI Secretion System. Cell Host Microbe 15:9–21.

Hooper, D. U., F. S. Chapin, J. J. Ewel, A. Hector, P. Inchausti, S. Lavorel, et al. 2005. Effects of Biodiversity on Ecosystem Functioning: A Consensus of Current Knowledge. Ecol. Monogr. 75:3–35.

Hunter, R. C., and T. J. Beveridge. 2005. Application of a pH-sensitive fluoroprobe (C-SNARF-4) for pH microenvironment analysis in Pseudomonas aeruginosa biofilms. Appl. Environ. Microbiol. 71:2501–10.

Ianiro, G., H. Tilg, and A. Gasbarrini. 2016. Antibiotics as deep modulators of gut microbiota: between good and evil. Gut 65:1906–1915.

Imperi, F., F. Tiburzi, and P. Visca. 2009. Molecular basis of pyoverdine siderophore recycling in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 106:20440–20445.

Inglis, R. F., J. M. Biernaskie, A. Gardner, and R. Kümmerli. 2016. Presence of a loner strain maintains cooperation and diversity in well-mixed bacterial communities. Proc. R. Soc. London B 283:20152682.

Jaeger, K.-E., K. Liebeton, A. Zonta, K. Schimossek, and M. T. Reetz. 1996. Biotechnological application of Pseudomonas aeruginosa lipase: efficient kinetic resolution of amines and alcohols. Appl. Microbiol. Biotechnol. 46:99–105.

Jamet, A., and X. Nassif. 2015. New players in the toxin field: polymorphic toxin systems in bacteria. MBio 6:e00285-15.

Jandhyala, S. M., R. Talukdar, C. Subramanyam, H. Vuyyuru, M. Sasikala, and D. Nageshwar Reddy. 2015. Role of the normal gut microbiota. World J. Gastroenterol. 21:8787–803.

Jayaseelan, S., D. Ramaswamy, and S. Dharmaraj. 2014. Pyocyanin: production, applications, challenges and new insights. World J. Microbiol. Biotechnol. 30:1159–1168.

Jiricny, N., S. P. Diggle, S. A. West, B. A. Evans, G. Ballantyne, A. Ross-Gillespie, and A. S. Griffin. 2010. Fitness correlates with the extent of cheating in a bacterium . J. Evol. Biol. 23:738–747.

Jones, C., L. Allsopp, J. Horlick, H. Kulasekara, and A. Filloux. 2013. Subinhibitory Concentration of Kanamycin Induces the Pseudomonas aeruginosa type VI Secretion System. PLoS One 8:e81132.

Joshi, F., G. Archana, and A. Desai. 2006. Siderophore cross-utilization amongst rhizospheric bacteria and the role of their differential affinities for Fe3+ on growth stimulation under iron-limited conditions. Curr. Microbiol. 53:141–147.

Joshi, F. R., S. P. Kholiya, G. Archana, and A. J. Desai. 2008. Siderophore cross-utilization amongst nodule isolates of the cowpea miscellany group and its effect on plant growth in the presence of antagonistic organisms. Microbiol Res 163:564–570.

Juhas, M., L. Eberl, and B. Tümmler. 2005. Quorum sending: the power of cooperation in the world of Pseudomonas. Environ. Microbiol. 7:459–471.

Julou, T., T. Mora, L. Guillon, V. Croquette, I. J. Schalk, and D. Bensimon. 2013. Cell-cell contacts confine public goods diffusion inside Pseudomonas aeruginosa clonal microcolonies. Proc. Natl. Acad. Sci. U. S. A. 110:12577–12582.

Kamada, N., S.-U. Seo, G. Y. Chen, and G. Núñez. 2013. Role of the gut microbiota in immunity and inflammatory disease. Nat. Rev. Immunol. 13:321–335.

Kaneko, Y., M. Thoendel, O. Olakanmi, B. E. Britigan, and P. K. Singh. 2007. The transition metal gallium disrupts Pseudomonas aeruginosa iron metabolism and has antimicrobial and antibiofilm activity. J. Clin. Invest. 117:877–888.

Keeney, K. M., S. Yurist-Doutsch, M.-C. Arrieta, and B. B. Finlay. 2014. Effects of Antibiotics on Human Microbiota and Subsequent Disease. Annu. Rev. Microbiol. 68:217–235.

Kelsic, E. D., J. Zhao, K. Vetsigian, and R. Kishony. 2015. Counteraction of antibiotic production and degradation stabilizes microbial communities. Nature 521:516–519.

Kerr, B., M. A. Riley, M. W. Feldman, and B. J. M. Bohannan. 2002. Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature 418:171–174.

Kim, H. J., J. Q. Boedicker, J. W. Choi, and R. F. Ismagilov. 2008. Defined spatial structure stabilizes a synthetic multispecies bacterial community. Proc. Natl. Acad. Sci. U. S. A. 105:18188–18193.

Kirkup, B. C., and M. A. Riley. 2004. Antibiotic-mediated antagonism leads to a bacterial game of rock-paper-scissors in vivo. Nature 428:694–696.

Kümmerli, R., and S. P. Brown. 2010. Molecular and regulatory properties of a public good shape the evolution of cooperation. Proc. Natl. Acad. Sci. U. S. A. 107:18921–18926.

Kümmerli, R., A. Gardner, S. A. West, and A. S. Griffin. 2009a. Limited dispersal, budding dispersal and cooperation: an experimental study . Evolution 63:939–949.

Kümmerli, R., A. S. Griffin, S. A. West, A. Buckling, F. Harrison, A. S. Griffin, S. A. West, A. Buckling, and F. Harrison. 2009b. Viscous medium promotes cooperation in the pathogenic bacterium Pseudomonas aeruginosa. Proc. R. Soc. B 276:3531–3538.

Kümmerli, R., N. Jiricny, L. S. Clarke, S. A. West, and A. S. Griffin. 2009c. Phenotypic plasticity of a cooperative behaviour in bacteria. J. Evol. Biol. 22:589–598.

Kümmerli, R., L. Santorelli, E. Granato, Z. Dumas, A. Dobay, A. S. Griffin, and S. A. West. 2015. Co-evolutionary dynamics between public good producers and cheats in the bacterium Pseudomonas aeruginosa. J. Evol. Biol. 28:2264–2274.

Lambiase, A., V. Raia, M. Del Pezzo, A. Sepe, V. Carnovale, and F. Rossano. 2006. Microbiology of airway disease in a cohort of patients with cystic fibrosis. BMC Infect. Dis. 6:4.

Lamont, I. L., P. A. Beare, U. Ochsner, A. I. Vasil, and M. L. Vasil. 2002. Siderophore-mediated signaling regulates virulence factor production in Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. U. S. A. 99:7072–7077.

Latunde-Dada, G. O. 2009. Iron metabolism: microbes, mouse, and man. BioEssays 31:1309–1317.

Lee, J., and L. Zhang. 2014. The hierarchy quorum sensing network in Pseudomonas aeruginosa. Protein Cell 6:26–41.

Leggett, H. C., S. P. Brown, and S. E. Reece. 2014. War and peace: social interactions in infections. Philos. Trans. R. Soc. B 369:20130365.

Leoni, L., N. Orsi, V. de Lorenzo, and P. Visca. 2000. Functional analysis of PvdS, an iron starvation sigma factor of Pseudomonas aeruginosa. J. Bacteriol. 182:1481–1491.

Lüdemann, H., I. Arth, and W. Liesack. 2000. Spatial changes in the bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. Appl. Environ. Microbiol. 66:754–62.

Lujan, A. M., P. Gomez, and A. Buckling. 2015. Siderophore cooperation of the bacterium Pseudomonas fluorescens in soil. Biol. Lett. 11:20140934–20140934.

Maravic, A., M. Skocibusic, M. Sprung, I. Samanic, J. Puizina, and M. Pavela-Vrancic. 2012. Occurrence and antibiotic susceptibility profiles of Burkholderia cepacia complex in coastal marine environment. Int. J. Environ. Health Res. 22:531–542.

Maravić, A., M. Skočibušić, M. Sprung, I. Samanić, J. Puizina, and M. Pavela-Vrančić. 2012. Occurrence and antibiotic susceptibility profiles of Burkholderia cepacia complex in coastal marine environment. Int. J. Environ. Health Res. 22:531–42.

Marmann, A., A. H. Aly, W. Lin, B. Wang, and P. Proksch. 2014. Co-cultivation--a powerful emerging tool for enhancing the chemical diversity of microorganisms. Mar. Drugs 12:1043–65.

Marvig, R. L., S. Damkiaer, H. Khademi, T. M. Markussen, S. Molin, and L. Jelsbak. 2014. Within-Host evolution of Pseudomonas aeruginosa reveals adaptation. MBio 5:1–8.

Marvig, R. L., L. M. Sommer, S. Molin, and H. K. Johansen. 2015. Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. Nat. Genet. 47:57–65.

Mashburn, L. M., A. M. Jett, D. R. Akins, and M. Whiteley. 2005. Staphylococcus aureus serves as an iron source for Pseudomonas aeruginosa during in vivo coculture. J. Bacteriol. 187:554–566.

Mathew, A., L. Eberl, and A. L. Carlier. 2014. A novel siderophore-independent strategy of iron uptake in the genus Burkholderia. Mol. Microbiol. 91:805–820.

McKenney, D., K. E. Brown, and D. G. Allison. 1995. Influence of Pseudomonas aeruginosa exoproducts on virulence factor production in Burkholderia cepacia: evidence of interspecies communication. J. Bacteriol. 177:6989–6992.

McNally, L., and S. P. Brown. 2015. Building the microbiome in health and disease: niche construction and social conflict in bacteria. Philos. Trans. R. Soc. London B 370:20140298.

Meredith, H. R., J. K. Srimani, A. J. Lee, A. J. Lopatkin, and L. You. 2015. Collective antibiotic tolerance: mechanisms, dynamics and intervention. Nat. Chem. Biol. 11:182–8.

Meyer, J. M., D. Hohnadel, and F. Halle. 1989. Cepabactin from Pseudomonas cepacia, a new type of siderophore. J Gen Microbiol 135:1479–1487.

Michel, L., N. Gonzalez, S. Jagdeep, T. Nguyen-Ngoc, and C. Reimmann. 2005. PchR-box recognition by the AraC-type regulator PchR of Pseudomonas aeruginosa requires the siderophore pyochelin as an effector. Microbiology 58:495–509.

Moradali, M. F., S. Ghods, and B. H. A. Rehm. 2017. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front. Cell. Infect. Microbiol. 7:39.

Mund, A., S. P. Diggle, and F. Harrison. 2017. The Fitness of Pseudomonas aeruginosa Quorum Sensing Signal Cheats Is Influenced by the Diffusivity of the Environment. MBio 8:e00353-17.

Murray, J. L., J. L. Connell, A. Stacy, K. H. Turner, and M. Whiteley. 2014. Mechanisms of Synergy in Polymicrobial Infections. 52:188–199.

Nadell, C. D., K. Drescher, and K. R. Foster. 2016. Spatial structure, cooperation and competition in biofilms. Nat. Rev. Microbiol. 14:589–600.

Nair, A. V., N. Joseph, K. Krishna, K. G. Sneha, N. Tom, K. Jangid, and S. Nair. 2015. A comparative study of coastal and clinical isolates of Pseudomonas aeruginosa. Brazilian J. Microbiol. 46:725–734.

Nguyen, A. T., M. J. O'Neill, A. M. Watts, C. L. Robson, I. L. Lamont, A. Wilks, and A. G. Oglesby-Sherrouse. 2014. Adaptation of iron homeostasis pathways by a Pseudomonas aeruginosa pyoverdine mutant in the cystic fibrosis lung. J. Bacteriol. 196:2265–2276.

Niehus, R., A. Picot, N. M. Oliveira, S. Mitri, and K. R. Foster. 2017. The evolution of siderophore production as a competitive trait. Evolution 71:1443–1455.

Novik, G., V. Savich, and E. Kiselev. 2015. An Insight Into Beneficial Pseudomonas bacteria. P. *in* M. M. Shah, ed. Microbiology in Agriculture and Human Health. InTech.

O'Brien, S., and J. L. Fothergill. 2017. The role of multispecies social interactions in shaping Pseudomonas aeruginosa pathogenicity in the cystic fibrosis lung. FEMS Microbiol. Lett. 364:1–10.

O'Brien, S., D. J. Hodgson, and A. Buckling. 2014. Social evolution of toxic metal bioremediation in Pseudomonas aeruginosa. Proc. R. Soc. London B 281:20140858.

Ochsner, U. A., A. I. Vasil, and M. L. Vasil. 1995. Role of the ferric uptake regulator of Pseudomonas aeruginosa in the regulation of siderophores and exotoxin A expression: purification and activity on iron-regulated promoters. J. Bacteriol. 177:7194–201.

Oliveira, N. M., E. Martinez-Garcia, J. Xavier, W. M. Durham, R. Kolter, W. Kim, and K. R. Foster. 2015. Biofilm formation as a response to ecological competition. PLoS Biol. 13:e1002191.

Özkaya, Ö., K. B. Xavier, F. Dionisio, and R. Balbontín. 2017. Maintenance of microbial cooperation mediated by public goods in single and multiple traits scenarios. J. Bacteriol. JB.00297-17.

Peters, B. M., M. A. Jabra-Rizk, G. A. O'May, J. W. Costerton, and M. E. Shirtliff. 2012. Polymicrobial interactions: impact on pathogenesis and human disease. Clin. Microbiol. Rev. Microbiol Rev 25:193–213.

Poltak, S. R., and V. S. Cooper. 2011. Ecological succession in long-term experimentally evolved biofilms produces synergistic communities. ISME J. 5:369–378.

Poole, K., and G. A. McKay. 2003. Iron acquisition and its control in Pseudomonas aeruginosa: many roads lead to Rome. Front. Biosci. 8:d661-686.

Preston, G. M. 2004. Plant perceptions of plant growth-promoting Pseudomonas. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 359:907–18.

Quinn, R. A., K. Whiteson, Y. W. Lim, J. Zhao, D. Conrad, J. J. LiPuma, F. Rohwer, and S. Widder. 2016. Ecological networking of cystic fibrosis lung infections. NPJ biofilms microbiomes 2:4.

Ratzke, C., J. S. Denk, and J. Gore. 2017. Ecological suicide in microbes. bioRxiv.

Ratzke, C., and J. Gore. 2017. Modifying And Reacting To The Environmental pH Drives Bacterial Interactions. bioRxiv.

Reen, F. J., M. J. Mooij, L. J. Holcombe, C. M. M. G. P. McGlacken, J. P. Morrissey, and F. O'Gara. 2011. The Pseudomonas quinolone signal (PQS), and its precursor HHQ, modulate interspecies and interkingdom behaviour. Fems Microbiol. Ecol. 77:413–428.

Reinhart, A., and A. Oglesby-Sherrouse. 2016. Regulation of Pseudomonas aeruginosa Virulence by Distinct Iron Sources. Genes. 7:126.

Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, et al. 2001. N -Acylhomoserine-lactone-mediated communication between Pseudomonas aeruginosa and Burkholderia cepacia in mixed biofilms. Microbiology 147:3249–3262.

Riley, M. A., and J. E. Wertz. 2002. Bacteriocins: Evolution, Ecology, and Application. Annu. Rev. Microbiol. 56:117–137.

Ross-Gillespie, A., Z. Dumas, and R. Kümmerli. 2015. Evolutionary dynamics of interlinked public goods traits: an experimental study of siderophore production in Pseudomonas aeruginosa. J. Evol. Biol. 28:29–39.

Ross-Gillespie, A., M. Weigert, S. P. Brown, and R. Kümmerli. 2014. Gallium-mediated siderophore quenching as an evolutionarily robust antibacterial treatment. Evol. Med. Public Heal. 2:18–29.

Ruhe, Z. C., D. A. Low, and C. S. Hayes. 2013. Bacterial contact-dependent growth inhibition. Trends Microbiol. 21:230–237.

Russell, A. B., R. D. Hood, N. K. Bui, M. LeRoux, W. Vollmer, and J. D. Mougous. 2011. Type VI secretion delivers bacteriolytic effectors to target cells. Nature 475:343–347.

Russell, A. B., S. B. Peterson, and J. D. Mougous. 2014. Type VI secretion system effectors : poisons with a purpose. 12:137–148.

Sadikot, R. T., T. S. Blackwell, J. W. Christman, and A. S. Prince. 2005. Pathogen–Host Interactions in *Pseudomonas aeruginosa* Pneumonia. Am. J. Respir. Crit. Care Med. 171:1209–1223.

Saha, R., N. Saha, R. S. Donofrio, and L. L. Bestervelt. 2013. Microbial siderophores: a mini review. J. Basic Microbiol. 53:303–317.

Sana, T. G., B. Berni, and S. Bleves. 2016. The T6SSs of Pseudomonas aeruginosa Strain PAO1 and Their Effectors: Beyond Bacterial-Cell Targeting. Front. Cell. Infect. Microbiol. 6:61.

Schaffer, K. 2015. Epidemiology of infection and current guidelines for infection prevention in cystic fibrosis patients. J. Hosp. Infect. 89:309–313.

Schalk, I. J., and G. L. A. Mislin. 2017. Bacterial Iron Uptake Pathways: Gates for the Import of Bactericide Compounds. J. Med. Chem. 60:4573–4576.

Schiessl, K., E. M.-L. Janssen, S. M. Kraemer, K. McNeill, and M. Ackermann. 2016. Magnitude and mechanism of siderophore-mediated competition in bacterial interactions. bioRxiv.

Schuster, M., D. J. Sexton, S. P. Diggle, and E. P. Greenberg. 2013. Acyl-homoserine lactone quorum sensing: from evolution to application. Annu. Rev. Microbiol. 67:43–63.

Schwab, U., L. H. Abdullah, O. S. Perlmutt, C. W. Davis, R. R. Arnold, R. James, et al. 2014. Localization of Burkholderia cepacia Complex Bacteria in Cystic Fibrosis Lungs and Interactions with Pseudomonas aeruginosa in Hypoxic Mucus. Infect Immun 82:4729–4745.

Scoffone, V. C., L. R. Chiarelli, G. Trespidi, M. Mentasti, G. Riccardi, and S. Buroni. 2017. Burkholderia cenocepacia Infections in Cystic Fibrosis Patients: Drug Resistance and Therapeutic Approaches. Front. Microbiol. 8:1592.

Servin, A. L. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28:405–440.

Seth, E. C., and M. E. Taga. 2014. Nutrient cross-feeding in the microbial world. Front. Microbiol. 5:350.

Silby, M. W., C. Winstanley, S. A. C. Godfrey, S. B. Levy, and R. W. Jackson. 2011. Pseudomonas genomes: diverse and adaptable. FEMS Microbiol. Rev. 35:652–680.

Sinumvayo, J. P., and N. Ishimwe. 2015. Agriculture and Food Applications of Rhamnolipids and its Production by Pseudomonas Aeruginosa. J. Chem. Eng. Process Technol. 6:223.

Smalley, N. E., D. An, M. R. Parsek, J. R. Chandler, and A. A. Dandekar. 2015. Quorum sensing protects Pseudomonas aeruginosa against cheating by other species in a laboratory co-culture model. J. Bacteriol. 197:3154–3159.

Smith, A. L., S. B. Fiel, N. Mayer-Hamblett, B. Ramsey, and J. L. Burns. 2003. Susceptibility Testing of Pseudomonas aeruginosa Isolates and Clinical Response to Parenteral Antibiotic Administration: Lack of Association in Cystic Fibrosis. Chest 123:1495–1502.

Sousa, A. M., and M. O. Pereira. 2014. Pseudomonas aeruginosa Diversification during Infection Development in Cystic Fibrosis Lungs-A Review. Pathogens 3:680–703.

Stefani S, Campana S, Cariani L, Carnovale V, Colombo C, Lleo MM, et al. 2017. Relevance of multidrug-resistant Pseudomonas aeruginosa infections in cystic fibrosis. Int. J. Med. Microbiol. 307:353–362.

Stephan, H., S. Freund, W. Beck, G. Jung, J. M. Meyer, and G. Winkelmann. 1993. Ornibactins-a new family of siderophores from Pseudomonas. Biometals 6:93–100.

Stites, S. W., M. W. Plautz, K. Bailey, A. R. O'Brien-Ladner, and L. J. Wesselius. 1999. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. Am. J. Respir. Crit. Care Med. 160:796–801.

Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, et al. 2000. Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. Nature 406:959–964.

Stubbendieck, R. M., and P. D. Straight. 2016. Multifaceted interfaces of bacterial competition. J. Bacteriol. 198:2145–2155.

Subramoni, S., and P. A. Sokol. 2012. Quorum sensing systems influence *Burkholderia cenocepacia* virulence. Future Microbiol. 7:1373–1387.

Suzuki, Y., S. Kajii, M. Nishiyama, and A. Iguchi. 2013. Susceptibility of Pseudomonas aeruginosa isolates collected from river water in Japan to antipseudomonal agents. Sci. Total Environ. 450–451:148–154.

Tashiro, Y., Y. Yawata, M. Toyofuku, H. Uchiyama, and N. Nomura. 2013. Interspecies Interaction between Pseudomonas aeruginosa and Other Microorganisms. Microbes Environ. 28:13–24.

Thomas, M. S. 2007. Iron acquisition mechanisms of the Burkholderia cepacia complex. Biometals 20:431–452.

Traxler, M. F., M. R. Seyedsayamdost, J. Clardy, and R. Kolter. 2012. Interspecies modulation of bacterial development through iron competition and siderophore piracy. Mol. Microbiol. 86:628–644.

Traxler, M. F., J. D. Watrous, T. Alexandrov, P. C. Dorrestein, and R. Kolter. 2013. Interspecies interactions stimulate diversification of the Streptomyces coelicolor secreted metabolome. MBio 4:e00459-13.

Trejo-Hernández, A., A. Andrade-Domínguez, M. Hernández, and S. Encarnación. 2014. Interspecies competition triggers virulence and mutability in Candida albicans-Pseudomonas aeruginosa mixed biofilms. ISME J. 8:1974–88.

Tyrrell, J., and M. Callaghan. 2016. Iron acquisition in the Cystic Fibrosis lung and potential for novel therapeutic strategies. Microbiology 162:191–205.

Tyrrell, J., N. Whelan, C. Wright, I. Sá-Correia, S. McClean, M. Thomas, and M. Callaghan. 2015. Investigation of the multifaceted iron acquisition strategies of Burkholderia cenocepacia. Biometals 28:367–380.

Vega, N. M., and J. Gore. 2014. Collective antibiotic resistance: mechanisms and implications. Curr. Opin. Microbiol. 21:28–34.

Vial, L., A. Chapalain, M. C. Groleau, and E. Deziel. 2011. The various lifestyles of the Burkholderia cepacia complex species: a tribute to adaptation. Environ. Microbiol. 13:1–12.

Visca, P., F. Imperi, and I. L. Lamont. 2007. Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. 15:22–30.

Visser, M. B., S. Majumdar, E. Hani, and P. A. Sokol. 2004. Importance of the ornibactin and pyochelin siderophore transport systems in Burkholderia cenocepacia lung infections. Infect. Immun. 72:2850–2857.

Vos, M., A. B. Wolf, S. J. Jennings, and G. A. Kowalchuk. 2013. Micro-scale determinants of bacterial diversity in soil. FEMS Microbiol. Rev. 37:936–954.

Wade, D. S., M. W. Calfee, E. R. Rocha, E. A. Ling, E. Engstrom, J. P. Coleman, and E. C. Pesci. 2005. Regulation of Pseudomonas quinolone signal synthesis in Pseudomonas aeruginosa. J. Bacteriol. 187:4372–80.

Wagner, S., R. Sommer, S. Hinsberger, C. Lu, R. W. Hartmann, M. Empting, and A. Titz. 2016. Novel Strategies for the Treatment of *Pseudomonas aeruginosa* Infections. J. Med. Chem. 59:5929–5969.

Wang, M., A. L. Schaefer, A. A. Dandekar, and E. P. Greenberg. 2015. Quorum sensing and policing of Pseudomonas aeruginosa social cheaters. Proc. Natl. Acad. Sci. U. S. A. 112:2187–2191.

Weaver, V. B., and R. Kolter. 2004. Burkholderia spp. alter Pseudomonas aeruginosa physiology through iron sequestration. J Bacteriol 186:2376–2384.

Weigert, M., and R. Kümmerli. 2017. The physical boundaries of public goods cooperation between surface-attached bacterial cells. Proc. R. Soc. B 284:20170631.

Weigert, M., A. Ross-Gillespie, A. Leinweber, G. Pessi, S. P. Brown, and R. Kümmerli. 2017. Manipulating virulence factor availability can have complex consequences for infections. Evol. Appl. 10:91–101.

West, S. A., A. S. Griffin, and A. Gardner. 2007. Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. J. Evol. Biol. 21:374–385.

West, S. A., A. S. Griffin, A. Gardner, and S. P. Diggle. 2006. Social evolution theory for microorganisms. Nat Rev Microbiol 4:597–607.

Westhoff, S., G. P. van Wezel, and D. E. Rozen. 2017. Distance-dependent danger responses in bacteria. Curr. Opin. Microbiol. 36:95–101.

Whitby, P. W., T. M. VanWagoner, J. M. Springer, D. J. Morton, T. W. Seale, and T. L. Stull. 2006. Burkholderia cenocepacia utilizes ferritin as an iron source. J. Med. Microbiol. 55:661–668.

Wilder, C. N., S. P. Diggle, and M. Schuster. 2011. Cooperation and cheating in Pseudomonas aeruginosa: the roles of the las, rhl and pqs quorum-sensing systems. ISME J. 5:1332–1343.

Winstanley, C., S. O'Brien, and M. A. Brockhurst. 2016. Pseudomonas aeruginosa Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. Trends Microbiol. 24:327–337.

Wolz, C., K. Hohloch, A. Ocaktan, K. Poole, R. W. Evans, N. Rochel, A. M. Albrecht-Gary, M. A. Abdallah, and G. Döring. 1994. Iron release from transferrin by pyoverdin and elastase from Pseudomonas aeruginosa. Infect. Immun. 62:4021–7.

World Health Organization. 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics.

Worlitzsch, D., R. Tarran, M. Ulrich, U. Schwab, A. Cekici, K. C. Meyer, et al. 2002. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J. Clin. Invest. 109:317–25.

Xiao, R., and W. S. Kisaalita. 1997. Iron acquisition from transferrin and lactoferrin by Pseudomonas aeruginosa pyoverdin. Microbiology 143:2509–2515.

Zehnder, A., and W. Stumm. 1988. Geochemistry and biogeochemistry of anaerobic habitats. Pp. 1–38 *in* A. Zehnde, ed. Biology of anaerobic microorganisms. John Wiley & Sons, Inc., New York.

Zhang, N., and Q.-S. He. 2015. Commensal Microbiome Promotes Resistance to Local and Systemic Infections. Chin. Med. J. (Engl). 128:2250–2255.

Acknowledgements

First of all, I want to thank Rolf for being the best supervisor I could have had. Thank you for giving me the opportunity to learn, for challenging me, for your patience and your never-ending support. I wish you only the best for your future, and hope you can give the same guidance to many more generations of PhD students.

I also want to thank Martin Ackermann and Leo Eberl for being in my committee and for your valuable input to make this thesis what it is now.

I am also extremely grateful to Adin, who introduced me into the mysterious world of R and statistics. Before you, I would have never thought how much fun it can be! I also want to thank stackoverflow to help me through the more frustrating times with R.

I could have never done my projects, if it were not for Gabriella Pessi, Yilei Liu and Martina Lardi, who have taught me everything I needed to know about RNA sequencing. I also want to thank everybody from Gabriella's group for the good lab atmosphere and time we had together.

I especially want to thank Judith Zingg for doing such a great job in organizing our graduate school, for the fun events, your support for myself and my fellow PhD students. I am glad you are there for us. Thank you also for giving me the opportunity to be a mentor for young PhD students.

This thesis would have, however, never been finished without Elisa, Elena, Chiara and Michael, who were there for me from the very beginning on. Thank you for being friends, colleagues, role-models, competition, motivators, therapists, drinking buddies, supporters, critics, travel buddies … I loved working with you! It was a very special time for me.

Thank you to Chiara, Selina, Alex, Jos, Subham and Santosh for joining our group, taking over and carrying on the good spirit.

I further want to thank my parents, my brother and my niece and nephew for their support, their critical questions that kept me thinking outside the box, and for reminding me that there is still a life outside of the university. I would not be at this point now, without you.

My family outside my family contributed to the success of my PhD time through all kinds of mischief. Thank you so much for always being there for me! Who would I be without you?

Last but not least, I want to express my biggest gratitude to Michi. Thank you for your support and understanding, for pushing me and for believing in me. And thank you for being there for the good times to come.

Curriculum vitae

Anne Leinweber

Date of birth: 13/03/1986
Nationality: German

Education

01/2013 –present	**PhD thesis at the University of Zürich, Switzerland** Department of Plant and Microbial Biology Supervisor: Prof. Dr. Rolf Kümmerli Title: "The Struggle for Iron: How it Affects Cooperation, Competition and Biodiversity in Bacterial Communities"
10/2009 - 06/2012	**Master of Science (M.Sc.) at the University of Konstanz, Germany** Study field: General Biology Master thesis: "Analysis of Quality Control Processes in *Caenorhabditis elegans*" in the group of Prof. Dr. Elke Deuerling
09/2006 – 05/2012	**Bachelor of Science (B.Sc.) at the University of Konstanz, Germany** Study field: General Biology Bachelor thesis in the group of Prof. Dr. Bernhard Schink
05/2005	**Walter-Gropius-Gymnasium in Dessau, Germany** Degree: Abitur

Scientific activities

Publications

Leinweber A, Weigert M, Kümmerli R (2017): The bacterium *Pseudomonas aeruginosa* senses and gradually responds to inter-specific competition for iron; (resubmission stage for *Evolution*)

Leinweber, A., R. Fredrik Inglis, and R. Kümmerli. 2017. Cheating fosters species co-existence in well-mixed bacterial communities. ISME J. 11:1179–1188.

Weigert, M., A. Ross-Gillespie, **A. Leinweber**, G. Pessi, S. P. Brown, and R. Kümmerli. 2017. Manipulating virulence factor availability can have complex consequences for infections. Evol. Appl. 10:91–101.

Conferences

URPP Evolution in Action retreat *Poster presentation*	Rheinfelden, Switzerland, 09/2016
116th General Meeting of the American Society of Microbiology (ASM Microbe 2016) *Poster presentation*	Boston, USA 06/2016
Jeff Gore Laboratory, Massachusetts Institute of Technology (MIT) *Oral presentation*	Cambridge, USA 06/2016

	15th Congress of the European Society for Evolutionary Biology (ESEB) *Oral presentation*	Lausanne, Switzerland 08/2015
	15th International Symposium on Microbial Ecology (ISME) *Poster presentation*	Seoul, South Korea 08/2014
	14th International Conference on Pseudomonas *Poster presentation*	Lausanne, Switzerland 09/2013
Workshops	Inclusive Fitness and Game Theory Organized by : Laurent Keller, Laurent Lehmann (University of Lausanne, Switzerland)	Arolla, Switzerland 06/2013
Secured fundings	URPP Evolution in Action grant; Project funding (15300 CHF)	2016
	ASM Student and Postdoctoral Travel Award (700 USD)	2016
Teaching	Teaching assistant in an annual practical student course (3.5 weeks) in Evolutionary Microbiology (BIO 291) *Planning and preparation of projects, supervising practical work and data analysis, grading*	2013 - 2016

Extracuricular activities

01/2015 - present	Professional and active **Mentor** for young PhD students of my graduate school „Life Science Zurich Graduate School“